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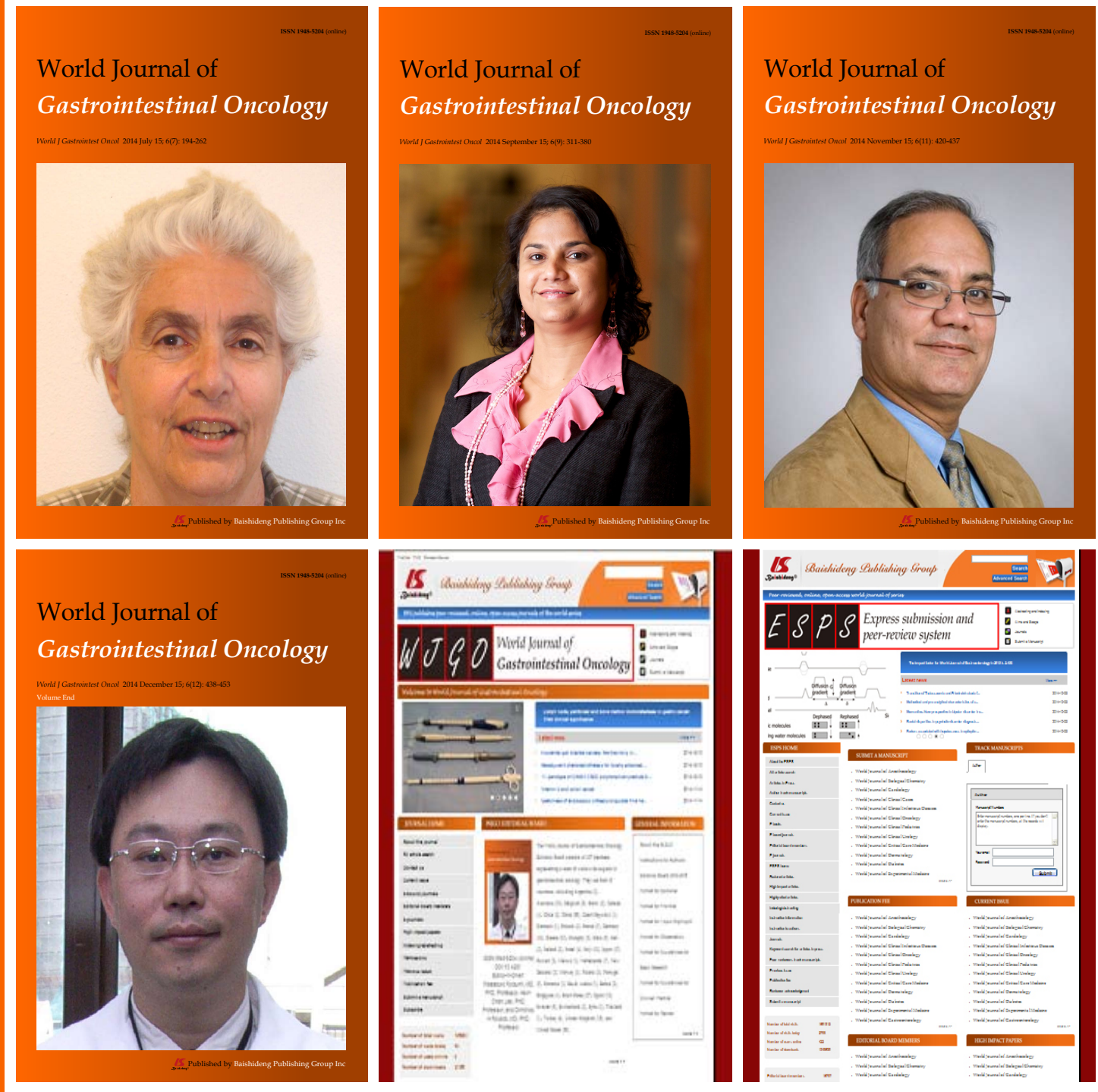

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AIM AND SCOPE

World Journal of Gastrointestinal Oncology (*World J Gastrointest Oncol*, *WJGO*, online ISSN 1948-5204, DOI: 10.4251) is a peer-reviewed open access academic journal that aims to guide clinical practice and improve diagnostic and therapeutic skills of clinicians.

WJGO covers topics concerning carcinogenesis, tumorigenesis, metastasis, diagnosis, prevention, prognosis, clinical manifestations, nutritional support, molecular mechanisms, and therapy of benign and malignant tumors of the digestive tract. The current columns of *WJGO* include editorial, frontier, diagnostic advances, therapeutics advances, field of vision, mini-reviews, review, topic highlight, medical ethics, original articles, case report, clinical case conference (Clinicopathological conference), and autobiography. Priority publication will be given to articles concerning diagnosis and treatment of gastrointestinal oncology diseases. The following aspects are covered: Clinical diagnosis, laboratory diagnosis, differential diagnosis, imaging tests, pathological diagnosis, molecular biological diagnosis, immunological diagnosis, genetic diagnosis, functional diagnostics, and physical diagnosis; and comprehensive therapy, drug therapy, surgical therapy, interventional treatment, minimally invasive therapy, and robot-assisted therapy.

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REVIEW

Approaches that ascertain the role of dietary compounds in colonic cancer cells

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Abstract

Preventive approaches against cancer have not been fully developed and applied. For example, the incidence of some types of cancer, including colon cancer, is highly dependent upon lifestyle, and therefore, amenable to prevention. Among the lifestyle factors, diet strongly affects the incidence of colon cancer; however, there are no definitive dietary recommendations that protect against this malignancy. The association between diet-derived bioactives and development of colonic neoplasms will remain ill defined if we do not take into account: (1) the identity of the metabolites present in the colonic lumen; (2) their concentrations in the colon; and (3) the effect of the colonic contents on the function of individual bioactives. We review two approaches that address these questions: the use of fecal water and *in vitro* models of the human colon. Compared to treatment with individual diet-derived compounds, the exposure of colon cancer cells to samples from fecal water or human colon simulators mimics closer the *in vitro* conditions and allows for more reliable studies on the effects of diet on colon cancer development. The rationale and the advantages of these strategies are discussed from the perspective of a specific question on how to analyze the combined effect of two types of bioactives, butyrate and polyphenol metabolites, on colon cancer cells.

Key words: Human colon model; Fecal water; Diet; Colon cancer; Prevention; Butyrate; Polyphenols; WNT signaling

Core tip: Studies on diet and colorectal cancer are in their infancy, and the relevance of many publications on the topic is questionable due to three problems: (1) there is uncertainty about which diet-derived compounds are present in the colon; (2) most studies have focused on individual bioactives; whereas, food intake results in complex metabolite mixtures; and (3) the physiological concentrations of many colonic bioactives are unknown. Here we discuss how the use of fecal water samples and *in vitro* models of human colon address these problems.

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INTRODUCTION

Within the past 100 years, the leading causes of death

have changed dramatically[1]. Approximately a century ago, the three leading causes of death were influenza and/or pneumonia, tuberculosis, and gastrointestinal (GI) infections. However, in 1997 less than 5% of the deaths were attributed to pneumonia, influenza, and human immunodeficiency virus infection; whereas, heart disease and cancers accounted for more than 50% of all deaths[2], In 2008, the American Cancer Society projected that soon cancer will become the leading cause of death worldwide[3], and the 2010 data for United States indicate almost equal number of deaths caused by heart disease and cancer (597689 *vs* 574743, respectively[4]). Recent projections of mortality and causes of death by the World Health Organization also support cancer as emerging leading cause of death in both, economically developed and developing countries[5]. How are these changes explained? The deaths from infectious diseases declined due to the implementation of childhood vaccinations, improvements in sanitation and hygiene, and the discovery of antibiotics. Except for the use of antibiotics, these approaches are classified as preventive measures. The more recent reduction of total cardiovascular death is also attributed to prevention; thus, massive educational efforts have raised the awareness of what constitutes a healthy lifestyle, and novel medications that control high blood pressure and cholesterol levels have been introduced into clinic. Therefore, the decreased deaths from infectious and heart diseases are mainly attributed to the development of preventive measures.

Unfortunately, the full power of prevention has not been applied in the battle against cancer. Presently, the focus is on cancer treatment, and as a result, billions of dollars are invested in drug development. The new arsenal of molecularly targeted anti-cancer drugs has raised hopes; however, it is increasingly clear that although "targeted" therapies prolong patients' lives, their benefit is limited in time by the inevitable acquisition of drug resistance. Combination therapies that incorporate conventional chemoradiation and molecularly targeted drugs might be the next step; however, the lesson from the past is that to obtain a significant victory against any disease, we need to emphasize on primary prevention.

Similar to the trend of personalized cancer treatment, future cancer prevention measures should be stratified by phenotype, genotype, and family history. Cancer prevention strategies could include, but not be limited to, the following: (1) monitoring of the patient's exposome (a set of biomarkers indicative of individual's exposure to cancer promoters[6]); (2) non-invasive imaging techniques that detect the earliest stages of abnormal growth; (3) reliable dietary, physical activity, and other lifestyle recommendations; and (4) vaccines that reduce the risk for specific cancers. In addition to developing future personalized prevention approaches, it is important to expand the existing prevention strategies that address some types of cancer as a public health issue affecting large populations (*e.g.*, educational approaches, influencing legislation, mobilizing communities). The present review focuses on the dietary approach to colorectal cancer (CRC) prevention, and addresses several problems that hinder the progress of this approach in terms of obtaining valid and unambiguous dietary recommendations.

There are over 140000 new cases of CRC and approximately 50000 CRC-related deaths a year in the United States[7]. A distinct characteristic of CRCs is that they develop slowly from benign adenomas: polyps larger than one centimeter in size have a 24% chance of progressing into carcinoma over a 20-year period[8]. The transition of benign adenomas into malignancies and the incidence of colonic neoplasms are modulated by diet-derived compounds[9]. However, studies on diet and CRC are in their infancy, and the relevance of many publications on the topic is questionable due to three problems: (1) there is uncertainty about which diet-derived compounds are present in the colon, and what their half-life; (2) most studies have focused on individual bioactives; whereas, food intake results in a complex mixture of metabolites that could modify each other's effect on neoplastic cells; and (3) the concentrations of many bioactives in the colon are unknown; whereas compounds, for which such information is available, have been frequently analyzed at levels exceeding physiological concentrations.

Here we review two approaches that address these problems, and discuss how these strategies solve a specific question on the interaction between two dietary bioactives: butyrate and polyphenol derivatives. Both bioactives affect the risk for CRC, and although there are other dietary compounds and mechanisms proposed to be protective against the malignancy, this review is limited to one example. Our objective is to highlight the methodologies that unravel the effects of multiple dietary bioactives on colonic cells, and not to comprehensively discuss all classes of dietary bioactives and their plausible physiological effects.

WNT/catenin signaling by butyrate

In 2011, the World Cancer Research Fund and the American Institute for Cancer Research upgraded the protective effect of fiber against colon cancer from "probable" to "convincing"[10] and this effect is attributed in part to the fermentation product of fiber in the colon, butyrate. Butyrate is a short-chain fatty acid (SCFA), the production of which enables the salvage of energy from dietary fiber that would be otherwise lost. It is estimated that SCFAs contribute to about 5%-15% of the total caloric requirements in humans[11]. Various tissues in the body can utilize SCFA for energy generation; however, butyrate is the preferred fuel for the colonic epithelial cells that derive about 70% of their energy from butyrate oxidation[12,13]. Butyrate is regarded as a healthy metabolite due to its positive influence on cell growth and differentiation, as well as its anti-inflammatory properties[12,14]. Butyrate also acts as an inhibitor of histone deacetylases (HDACi). Its colonic concentration is between 2 and 10 mmol[15] and at these levels, butyrate induces apoptosis in most CRC cells *in vitro*. We have provided evidence that this effect is in part due to the ability of butyrate to hyperactivate the WNT/catenin signaling pathway, and several synthetic

HDACis mimic the effect of butyrate on the WNT pathway and apoptosis[16,17]. The hyperactivation of WNT/catenin signaling by HDACis takes place only in colonic neoplastic cells with mutations in the pathway, and such mutations are detected in 80% of the sporadic colon cancers[18-20]. This finding is in agreement with observations that moderate levels of oncogene activities support cancer development; however, hyperactivation of oncogenic functions may result in cell death and senescence[21]. Therefore, WNT/catenin signaling is not "oncogenic" under all conditions, and sometimes its activation correlates with less aggressive cancer phenotypes[22].

Polyphenols as biological food constituents

The intake of fiber (the most important source of butyrate in the colon) is usually associated with that of other bioactive ingredients; for example, many fiber-rich foods are a source of polyphenols (*e.g.,* cereals, fruit, and vegetables). The drinks that accompany our meals further increase the complexity of bioactives: wine, fruit juices, cocoa, tea, and coffee are all rich in polyphenols. The two main classes of dietary polyphenols are the flavonoids and the phenolic acids. In *in vitro* experiments, the flavonoids are powerful antioxidants; however, this activity is exhibited at concentrations exceeding the levels achievable *in vivo*. Thus, after consumption of 10-100 mg of a single compound, the maximum plasma levels of individual flavonoids are approximately 1-3 mol[23,24]. In addition, due to host metabolism the *in vivo* half-life of the precursor polyphenols is short due to their rapid conversion into metabolites, all of which exhibit diminished antioxidant activity[24-26]. More recent studies indicate that at physiological concentrations, polyphenols and their metabolites modulate cell signaling pathways[27], and exhibit anti-inflammatory activity through inhibition of COX-2 protein levels, prostanoid biogenesis, or pro-inflammatory cytokine production[28-31]. Polyphenol metabolites also exhibit anti-proliferative effect on neoplastic cells[32,33], thus, similar to butyrate, some polyphenols and their microbial metabolites exhibit a CRC protective role. For example, quercetin, a flavonol found in citrus fruit, buckwheat, and onions, suppresses the formation of aberrant crypt foci and induces apoptosis in preneoplastic human colonocytes[34,35]. Caffeic acid esters present in propolis are potent inhibitors of human colon adenocarcinoma cell growth, carcinogen-induced biochemical changes, and preneoplastic lesions in the rat colon[36,37]. A CRC-preventive role has also been reported for isoflavons, curcumin, and tea polyphenol in green tea, (-)-epigallocatechin-3-gallate (EGCG)[38].

Synergistic or antagonistic effects of butyrate and polyphenols?

Since the intake of dietary fiber is frequently accompanied by that of polyphenols, it is logical to investigate whether the effect of butyrate on WNT/catenin signaling and apoptosis in CRC cells are modified by polyphenols and their metabolites. Presently, the combined effects of butyrate and polyphenol metabolites on WNT/catenin signaling are unknown; however, there have been reports on the modulation of WNT/catenin signaling by polyphenols. For example, polymeric black tea polyphenols inhibit 1,2-dimethylhydrazine-induced colorectal tumorigenesis in rats, and the researchers proposed that this effect is mediated by suppression of WNT/catenin signaling[39]. EGCG suppresses WNT/catenin transcriptional activity in HCT-116 CRC cells at concentrations of 100-200 mol, which are unachievable *in vivo*[40]. However, at physiologically relevant concentration of 0.5 mol[23,24,32], EGCG inhibits the enzyme glycogen synthase kinase-3 beta (GSK-3beta)[41]. This inactivation of GSK-3beta should result in accumulation of transcriptionally active Ser-37/Thr-41-dephosphorylated beta-catenin, and increased WNT transcriptional activity[42,43]. Polyphenol-rich apple juice extract, as well as the free aglycon phloretin and the flavonol quercetin, also inhibit GSK-3beta in *in vitro* assays[44]. In agreement with this inhibitory effect on the enzyme, quercetin at 10 mol increases WNT/catenin transcriptional activity[41]. The interpretation of these findings is difficult due to the fact that the bioavailability of the compounds has not been taken into account, or is unknown. In addition, polyphenols are biochemically transformed or completely fermented by the gut microbiota to metabolites with a modified biological activity, as discussed below. The inhibition of GSK-3beta by some polyphenols indicates that these compounds may synergize with butyrate in its effect on WNT/beta-catenin signaling. Furthermore, similar to butyrate, some polyphenols and their metabolites inhibit histone deacetylases (HDACs). Thus, fermentation of polyphenol-rich apple juice extracts with human fecal slurry revealed that polyphenol metabolites have a HDAC inhibitory function[45]. Metabolites of polyphenols in the colon, such as *P* Coumaric acid, 3-(4-OH-phenyl)-propionate, and caffeic acid also exhibit HDAC inhibitory function in *in vitro* assays with nuclear extracts from HT-29 human CC cells[46]. Therefore, similar to butyrate[16,17], polyphenol metabolites with HDAC inhibitory function may protect against CC *via* stabilization of beta-catenin and hyperinduction of WNT/beta-catenin signaling. Despite these data, the question of how polyphenols and their metabolites modulate the effects of butyrate on colonic neoplastic cells has remained unanswered. Several problems hinder the progress of the studies: there is little knowledge about the polyphenol derivatives present in the colon, their physiological concentrations, and how the colonic content modulates the functions of the bioactives. The main colonic species might be the polyphenol aglycones and their derivatives: phenolic and non-phenolic aromatic acids. The deglycosylation of polyphenols is catalyzed by microbial beta-glucosidases in the small intestine and primarily the colon, and this process results in aglycone forms that are more absorbable[47]. After absorption in the intestinal cells, the aglycones are metabolized to conjugates of glucuronate and sulfate, which are the major forms in plasma and urine[47]. However, these conjugates have not been detected in the colon, most likely due to the hydrolase activity of the GI microbiota[48-50].

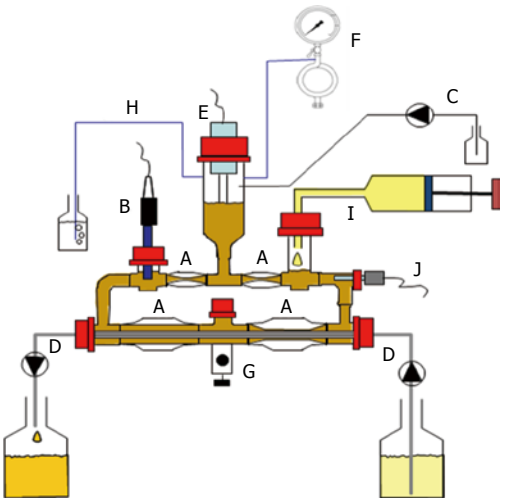

Figure 1 TIM-2 is a validated, computer-controlled system that simulates the human colon. The model consists of glass units with a flexible wall inside (A); Peristaltic movements, achieved by pumping warm water into the space between the glass unit and the flexible walls at regular intervals, simulate peristaltic movements and allow the lumen to be mixed and transported through the loop-shaped system. The system is kept at body temperature (37 ℃). To simulate the pH in the proximal colon, the pH is set at 5.8 and controlled (B) and adjusted by secretion of 2 mol/L NaOH into the system (C). A dialysis membrane consisting of semi-permeable hollow fibres is placed in the lumen (D). Water and fermentation products are removed from the lumen through the dialysis system, thereby maintaining physiological concentrations of microbial metabolites and preventing accumulation of metabolites to toxic levels. Furthermore the model contains an inlet system for the delivery of food (I) and a level sensor to control (E) a constant volume of the luminal content. The system was kept anaerobic by flushing with nitrogen gas (F), which allowed for the growth of a dense, complex microbiota, comparable to that found in the proximal colon of humans. A: Peristaltic compartments containing fecal matter; B: pH electrode; C: Alkali pump; D: Dialysis liquid circuit with hollow fibre membrane; E: Level sensor; F: N2 gas inlet; G: Sampling port; H: Gas outlet; I: "Ileal efflux" container; J: Temperature sensor.

Use of fecal water

The problems listed above are not specific to our example on the combined effect of dietary butyrate and polyphenols on colon cancer cells, as they represent a stumbling block for all studies aimed at characterization of the effects of dietary bioactives. To date, there are two approaches that address these problems: (1) performing analyses with the aqueous phase of feces (fecal water); and (2) utilizing *in vitro* GI models. The first approach is justified by the fact that the colonic epithelium is exposed to the fecal matter *in vivo*[48,51-54] and that fecal water affects the growth of colonocytes more effectively than components of the solid phase of feces[52,53]. Gas chromatography and mass spectrometry analyses of the fecal water of healthy volunteers have identified and quantified the flavonoids and their derivatives in the colon[48]. In these samples, the most prevalent flavonoids were naringenin, quercetin, formononetin, catechin, epicatechin, isorhamnetin, apigenin, and kaempferol, and they were detected at mean concentrations of 1.2, 0.63, 0.17, 0.14, 0.11, 0.10, 0.07 and 0.05 mol, respectively. All polyphenols and derivatives exhibited daily fluctuations in the same individual, and the most prevalent flavonoids naringenin, quercetin, and formononetin reached a maximum concentration of 4.04, 1.30, and 0.84 mol, respectively. Colonic derivatives of the flavonoids in the colon were detected at concentrations up to two orders of magnitude higher than these of their precursors; thus, the total monophenolic acids reached up to 740.7 mol and the total nonphenolic aromatic acids, 1.5 mmol[48]. Recent analyses of fecal water have confirmed the prevalence of the phenolic and non-phenolic aromatic acids in fecal water[28,54]. Therefore, our question of how polyphenols and their metabolites modulate the activity of butyrate may need to be re-stated to how the activity of butyrate is affected by high levels of monophenolic and nonphenolic acids.

In vitro models that mimic the human colon

The combined effect of butyrate and polyphenol metabolites on neoplastic cells, however, is even more complex. The combined effect could be modified by the presence of additional metabolites, as the intake of any diet results in a complex mixture of compounds in the colon. The physiological properties of diet-derived mixtures could be analyzed with *in vitro* models of the human GI tract, and one such model has been developed by TNO in the Netherlands[55]. This system closely mimics the physiological conditions in the GI tract, as established in numerous validation studies[32,56-67]. The GI system is composed of two separate models: TIM-1 that simulates the stomach and the small intestine (not further discussed here), and TIM-2 that simulates the colon[68] and contains compartments with a high density, metabolically active microbiota of human origin. The physiological conditions of the large intestine that are simulated include pH, anaerobiosis and gradual intake of pre-digested meal compounds coming from the small intestine (Figure 1). Physiological amounts of microorganisms in the TIM-2 model are maintained *via* dialysis mechanism. This mechanism takes up electrolytes and microbial metabolites, and ensures that the concentrations of these remain at physiological levels, preventing inhibition of the microbiota by metabolites. The *in vitro* GI system permits the use of an intestinal microbiota from different enterotypes and the comparison between various donors, *e.g.,* healthy *vs* diseased, lean *vs* obese[69]. The technology also allows for controlled analyses on the colonic outputs from various diets. Thus, entire meals representative of different types of diet can be "fed" to the GI model[60] and the resulting real-time fermented samples from the TIM-2 compartments can be tested on neoplastic and normal colonic cells *in vitro*[32]. Although fecal water from human subjects could be used for similar studies, there are several problems associated with this approach: inter-individual differences in metabolic rates and colonic microbiota, noncompliance with diet, preferential absorption of some compounds by the colonocytes, and the impossibility of acquiring samples from different locations of the human GI tract (*e.g.,* pre-colon, proximal colon). The last point is important, since metabolite concentrations change along the colon[11]. Using the *in vitro* GI system has several advantages: it is computer-controlled, allowing standard-

ization of the experiments, it is cheaper than clinical or animal trials, and it does not have the ethical constraints associated with animal and human subject studies. Furthermore, sampling from various locations along the GI tract and at different time points enables kinetic studies of the microbial metabolism of dietary components. The application of the *in vitro* gut approach can facilitate the design of functional foods and dietary supplements that decrease CRC incidence. For example, utilizing the *in vitro* GI system, Gao *et al*[32] discovered that tea, citrus fruit, and soy flavonoids are metabolized in the colon to a few phenolic and aromatic acids, therefore ascertaining the exact compounds that should be screened for effects on CRC cells.

In addition to the studies performed with the computerized human gut TIM-2, there are numerous reports on simpler colon simulators, and the function of some of these has been validated by chemical and microbiological measurements of the intestinal contents of human sudden death victims[70]. These models are fermentation systems that closely reproduce the proximal and distal human colon in terms of physicochemical parameters by utilizing a number of different vessels and continuous or semi-continuous culturing modes[71]. For example, a two-stage compound continuous culture models consisting of a proximal vessel (with lower pH) and a distal vessel (with higher pH) inoculated with human feces have been used to evaluate how various nutrients and supplements affect genotoxicity of the colonic environment and the populations of human gut bacteria[72,73]. Continuous culture models have been applied to analyses of how certain prebiotics affect the fecal metabolite profile, the survival of probiotics, and the interactions between various colonic microbial populations[74-77]. The effect of retention time (colonic transit time) on the catabolism of organic sources of carbon and nitrogen have been analyzed by a three-stage continuous culture model, which revealed that the majority of carbohydrate breakdown and SCFA production takes place in the proximal part of the colon (in the first vessel); whereas, formation of branched-chain fatty acids and phenolic compounds, occurs primarily in the distal part (mimicked by vessels 2 and 3)[70]. Other three-stage continuous culture colonic models inoculated with human fecal material were utilized to quantitate bacteria and evaluate the fermentability of oligosaccharide sources[78,79]. Four-stage semicontinuous model systems of the human colon, in which the four compartments mimic the conditions of the ascending, transverse, descending and sigmoid colon, have been employed to investigate the effects of probiotics, prebiotics, and various synbiotic combinations[80-82].

Applied to our question of whether the apoptotic and WNT signaling-modulating functions of butyrate are affected by diet-derived polyphenol compounds and their metabolites, the strategy utilizing *in vitro* gut models would be a reliable approach.

Thus, digesta samples from *in vitro* fermentation systems or the computerized TIM-2 model, instead of individual compounds, should be used to analyze the effects of various diets on colonic cancer cells.

Screening for dietary components that increase butyrate production by the colonic microbiota

TIM-2 allows determining the potential of dietary fibers to produce butyrate by the microbiota under physiological conditions. In an extensive study comparing 17 fibers Maathuis *et al*[57] showed varying levels of butyrate production for each fiber, with the highest production resulting from pullulan. Interestingly, this fiber also produced high levels of lactate, an intermediate intestinal metabolite that accumulates when there is a fast fermentation of a substrate. Lactate is usually converted into propionate[83] and butyrate[84] and through cross-feeding between different members of the microbiota. Butyrate is also produced through cross-feeding from acetate; thus, using ^{13}C-starch Maathuis *et al*[58] have shown that cross-feeding between *Ruminococcus bromii* and *Eubacterium rectale* results in production of butyrate from acetate[56]. Similarly, using ^{13}C-labeled galacto-oligosaccharides it was shown that lactate, produced by *Bifidobacteria* and *Lactobacilli*, was converted into butyrate. These two cross-feeding reactions in the colon could be quantified[85,86] and an *in silico* model can be used to predict production of the various SCFA by the colonic microbiota.

Analyses of human fecal samples also allow for focused analyses of how dietary changes affect butyrate levels in different individuals. Thus, considerable variations in fecal butyrate concentrations have been detected among individuals consuming resistant starch or nonstarch polysaccharides in a randomized cross-over study[87]. McOrist and colleagues reported that intake of resistant starch overall increases butyrate concentrations in most, but not all, individuals[87].

Analyses with a semi-continuous colonic simulator revealed that *Lactobacillus acidophilus* NCFM™ in combination with lactitol increases the numbers of *Bifidobacteria*, and stimulates synergistically the production of butyrate[82]. Similar colonic simulation system consisting of three vessels and inoculated with fecal slurry from healthy nonmethane producing donors established the parameters of SCFA production, including this of butyrate[70].

Use of in vitro models to study the microbial metabolism of polyphenols in the colon

Approximately 90%-95% of dietary polyphenols are not absorbed in the small intestine and reach the colon intact[88]. In the case of monomeric units, studies performed with ileostomy patients have shown that almost 70% of the ingested monomeric flavanols are accumulated in the colon, with 33% corresponding to the intact parent compounds[89]. As mentioned above, the major colonic metabolites of the polyphenols are phenolic acids. Thus, (epi) catechin and the monomeric units of procyanidins are degraded into several phenolic acids, namely various substituted phenylvaleric, phenylpropionic, phenylacetic, benzoic, and hippuric acid[90-92]. Additional metabolites from catechin and epicatechin such as 5-(3,4-dihydroxyphenyl)-γ-valerolactone and 5-(3-hydroxyphenyl)-γ-valerolactone

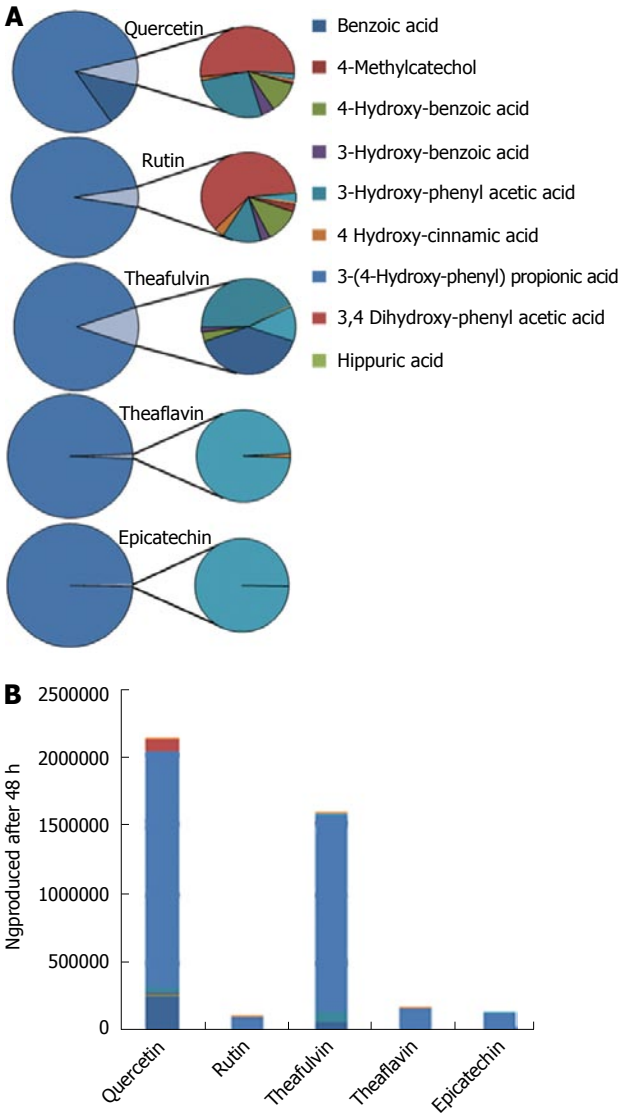

Figure 2 Cumulative production of 'simple' phenolic metabolites after 48 h fermentation of different polyphenols in TIM-2. At time zero a single shot of individuals polyphenols (1 microgram in dimethyl sulfoxide) was introduced into TIM-2 throigh the sampling port (Figure 1G). At regular intervals for the next 48 h samples were taken from the lumen and dialysate and analyzed using LC-MS for the microbial metabolites generated by the gut microbiota. The ratio (bar graph, in percentage) and absolute cumulative production (B; in ng) at t = 48 h of microbial metabolites after fermentation in TIM-2 were subsequently calculated and compared amongst the different polyphenols.

have been identified in man[90,91,93].

Under physiological conditions, the monomeric polyphenols are fermented rapidly; therefore, it is unknown whether these compounds have sufficient half-life to affect colonic (neoplastic) tissue from the luminal side, or whether the resulting microbial metabolites exert a stronger biological effect. Studies with human gut models can facilitate the answer to this question. In unpublished studies with TIM-2, we have observed that the same microbiota metabolizes different polyphenols to different low-molecular weight aromatic acids with variable hydroxylation profile and length of the aliphatic side chain (Figure 2). The number of produced microbial metabolites ranged from two (for epicatechin) to 12 (for quercetin). Even glycosylation of the polyphenols (*e.g.,* quercetin versus rutin) affected the production of microbial metabolites, likely because different groups of colonic microorganisms ferment quercetin and rutin. Thus, compared to other polyphenols, fermentation of rutin resulted in decreased proportion of benzoic acid and other metabolites (Figure 2), as well as an about 20-fold lower absolute amount of metabolites.

Analyses with colonic simulators allow for the detection of new colonic metabolites. In urine, the most frequent metabolite found after polyphenol ingestion is hippurate. This metabolite, a conjugate of benzoic acid and glycine, is considered to be produced by co-metabolism of the host and the microbiota. Benzoic acid is produced from the phenolic acids produced by the microbiota, and the glycine is thought to be coupled to benzoic acid in the liver. However, in the *in vitro* human gut TIM-2, which lacks the host metabolism component, we have shown that hippurate is also produced, indicating that the colonic microbiota by itself produces the metabolite (Figure 2).

Studies with colonic models could also address the question on the half-life of monomeric flavanols. For example, in studies on the dimeric forms of chocolate procyanidins Appeldoorn *et al*[90] have shown that the human microbiota produce several metabolites: 2-(3,4-dihydroxyphenyl)acetic acid, 2-(3-hydroxyphenyl)acetic acid, 2-(4-hydroxyphenyl)acetic acid and 3-(3-hydroxyphenyl)propionic acid, as well as various hydroxylated phenylvaleric acids, phenylvalerolactones,and 1-(3',4'-dihydroxyphenyl)-3-(2',4',6'-trihydroxyphenyl)propan-2-ol. The researchers also indicated that the formation of smaller metabolites was due to the direct degradation of dimers instead of cleavage of the monomeric form as previously assumed[90]. It is still possible that some procyanidin dimers are converted into monomeric flavanols before being fermented into phenolic acids; however, monomeric flavanols are rapidly metabolized, and therefore their presence is difficult to analyze[94].

Finally, phenolic acids produced from flavanols by the colonic microbiota significantly inhibit pathogenic human intestinal bacteria, such as *Clostridium perfringens*, *Staphylococcus aureus*, *E. coli*, and *Salmonella,* while exhibiting a much lower inhibition of commensal bacteria and probiotics, *Clostridium*, *Bifidobacterium* and *Lactobacillus*[95,96]. One mechanism mediating this activity is the destabilization of the outer membrane of *Salmonella* species[97]. Since changes in microbiota composition influence the production of butyrate from dietary fiber, the combined effects of polyphenols and fibers need to be thoroughly investigated in colonic simulator systems that include the naturally occurring colonic microorganisms.

CONCLUSION

Analyses on individual bioactives pinpoint their molecular targets in cells; however, such studies (1) should utilize physiological concentrations of the compounds; and (2)

should be accompanied by analyses with colonic digesta from different diets, since the activity of individual metabolites is likely modified by the complex colonic milieu. Such studies can be facilitated by the use of artificial GI systems and fecal water samples. This type of analyses will assist the design of functional foods and/or dietary supplements with CRC-preventive role.

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REVIEW

Antidepressant fluoxetine and its potential against colon tumors

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Key words: Fluoxetine; Colon cancer; Cancer therapy; Tumor metabolism

Core tip: It is currently thought that aerobic glycolysis is key for understanding cell survival in the hostile tumor microenvironment. Then, the antidepressant fluoxetine (FLX) has been shown to reduce colon tumor growth in animals and colon cancer incidence in humans. Here, we explore new perspectives of FLX reducing the development of colon tumors through a blockage in tumor metabolism. This perspective review is based on our current unpublished experimental dataset which shows FLX as a potential co-chemotherapeutic agent for colon cancer therapy.

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Abstract

Colon cancer is one of the most common tumors worldwide, with increasing incidence in developing countries. Patients treated with fluoxetine (FLX) have a reduced incidence of colon cancer, although there still remains great controversy about the nature of its effects. Here we explore the latest achievements related to FLX treatment and colon cancer. Moreover, we discuss new ideas about the mechanisms of the effects of FLX treatment in colon cancer. This leads to the hypothesis of FLX arresting colon tumor cells at the at G_1 cell-cycle phase through a control of the tumor-related energy generation machinery. We believe that the potential of FLX to act against tumor metabolism warrants further investigation.

INTRODUCTION

Colon cancer is one of the most common human malignancies worldwide and much effort has been applied to understand its development. The discovery of new therapeutical strategies or potential co-therapeutical agents against it might reduce the suffering of millions of people. A growing body of evidence suggests that the use of fluoxetine (FLX), an antidepressant belonging to the selective serotonin reuptake inhibitors (SSRIs), may be related to a reduced colon cancer incidence. However, its activity is not completely understood and potential new mechanisms are unknown to date.

Here, we discuss our recent published and unpublished data regarding the activity of FLX against colon cancer. This review takes a fresh view of the material, mainly of how FLX acts to block malignant metabolism,

reducing colon tumors.

COLON CANCER

The American Cancer Society estimates the number of new cases and expected deaths for cancer in the United States every year[1]. About 1.5 million cases and 569490 deaths of cancer were expected in 2010. This ranked colon cancer as the third most common cancer in the United States, with almost 50000 deaths per year[1,2]. In this year, it is expected that more than 143460 patients will be newly diagnosed with colon cancer in the United States[3]. Although survival has increased during the 5 years after diagnosis[2], a 60% increase for newly diagnosed cancer cases is projected for developing countries until 2030[4]. This highlights colon cancer as one of the major human malignancies worldwide and a great challenge for cancer therapy[5-7].

Adenoma-adenocarcinoma sequence model

The adenoma-adenocarcinoma sequence model is the most well-known and accepted hypothesis for the development of colon cancer[8]. It is thought that a sequence of mutations of the epithelial stem cell niche induces the development of colon tumors through different stages, such as initiation, promotion and progression[8]. Initiation is known as an irreversible step, where mutations in one or two gatekeeper genes occur in a single cryptal stem cell. This will then disrupt cell proliferation, leading to the expansion of malignant clones, a process termed promotion[9,10]. Mutations are thought to derive from cell exposure to carcinogenic compounds which directly attack the DNA or lead to increased oxidative stress (OS) with the generation of reactive oxygen species (ROS), which would then attack the DNA basis inducing mutations[11,12]. Clever's research group has elegantly generated *Lgr5*-$^{EGFP\text{-}IRES\text{-}creERT2}$/*Apc*$^{flox/flox}$ mice, which have a stem cell-specific knockin reporter for tamoxifen-inducible loss of the adenomatous polyposis coli (APC) sequence, and found that this genetic deletion in epithelial stem cells leads to their transformation within days, which was due to β-catenin accumulation[13]. This further supports the idea that a monoclonal propagation of acquired stem cell mutations occurs during the initial steps of colon carcinogenesis[9]. The manifestation of mutations in colon epithelia seems to be closely related to hyperproliferation[13-15]. In fact, mutations in the *APC* gene sequence at cryptal stem cell niches activate hyperproliferation due to an increase in β-catenin transcriptional activity which blocks p53 activity[15-17].

Subepithelial cells and their role in carcinogenesis

The cancer-enhancing activity of the microenvironment has been a matter of discussion since recent reports showed that disrupting key genetic sequences in stromal cells abrogates epithelial homeostasis, which then induces tumors[18-20]. An elegant report has specifically shown that epithelial tumors have arisen in forestomach after disrupting the transforming growth factor-β (TGF-β) signaling within the subepithelial compartment[21]. Previous studies had already shown that the subepithelial TGF-β signaling has tumor promoting potential on epithelial cells, due to its control over proliferation[22,23]. Nevertheless, under inflammatory conditions, subepithelial cells seem to be able to transform epithelial progenitor cells towards malignancy[20]. These ideas have actually been applied to colon carcinogenesis, confirming the malignant participation of subepithelial cells in the development and manifestation of colon tumors[20,24,25].

TUMOR METABOLISM

Hyperproliferation enables the clonal expansion of mutated cells, which further drives tumor growth[14,15,17,26-29]. For this, tumor cells require: high and fast adenosine-5'-ATP generation; a tightened maintenance of the cell redox status to overcome the stressful tumor microenvironment; and enhanced biosynthesis of macromolecules. Basically, tumor cells shift their energy generation machinery from oxidative phosphorylation to an aerobic-glycolytic metabolism[30,31]. This allows tumor cells to keep a high ATP generation and at the same time to avoid the negative feedback regulation from overusing glycolysis, which would otherwise activate metabolic and cell-cycle inhibitors, such as p53[30]. This was extensively discussed by Cairns *et al*[31]. Specifically, glycolysis-related mechanisms enhance the synthesis of nucleotides and DNA repair[30,31]. However, high proliferation enlarges the distance between cells and microvessels, which reduces the oxygen and nutrient supplies to the cells and creates a hypoxic microenvironment. While hypoxia generally promotes the expression of growth factors, inducing neovascularization, hypoxic areas in tumors may persist due to the chaotic and malformed structures of tumoral vessels and microvessels[14,32-34].

Moreover, hypoxic tumor cells are known to use glycolysis in order to increase energy generation (Figure 1). This requires an over-activation of glucose transporters (*i.e.*, GLUT1), lactate transporters (*i.e.*, MCT4) and lactate dehydrogenase A (LDH-A) through the hypoxia-inducible factor 1 (HIF-1) transcriptional activity. By inhibiting the degradation of HIF-1, a transcription factor which upregulates the glycolysis-related molecular activities, tumor cells increase the conversion of pyruvate to lactate[32,35]. Because tumor cells would then suffer from the hypoxia-induced and glycolysis-related acidosis, they alkalinize their intracellular pH (ipH) on their way to survival and proliferation. This is achieved *via* hyperactivation of HIF-1 activity, which enhances the hydration of carbon dioxide to bicarbonate by the catalytic activity of carbonic anhydrase Ⅸ and Ⅻ enzymes and promotes the activity of MCT-4 to extrude lactate and H^+ ions, both supporting an ipH alkalinization[32,36]. Overall, tumor cells undergo deep metabolic changes on their way to survival in the stressful tumoral microenvironment[31].

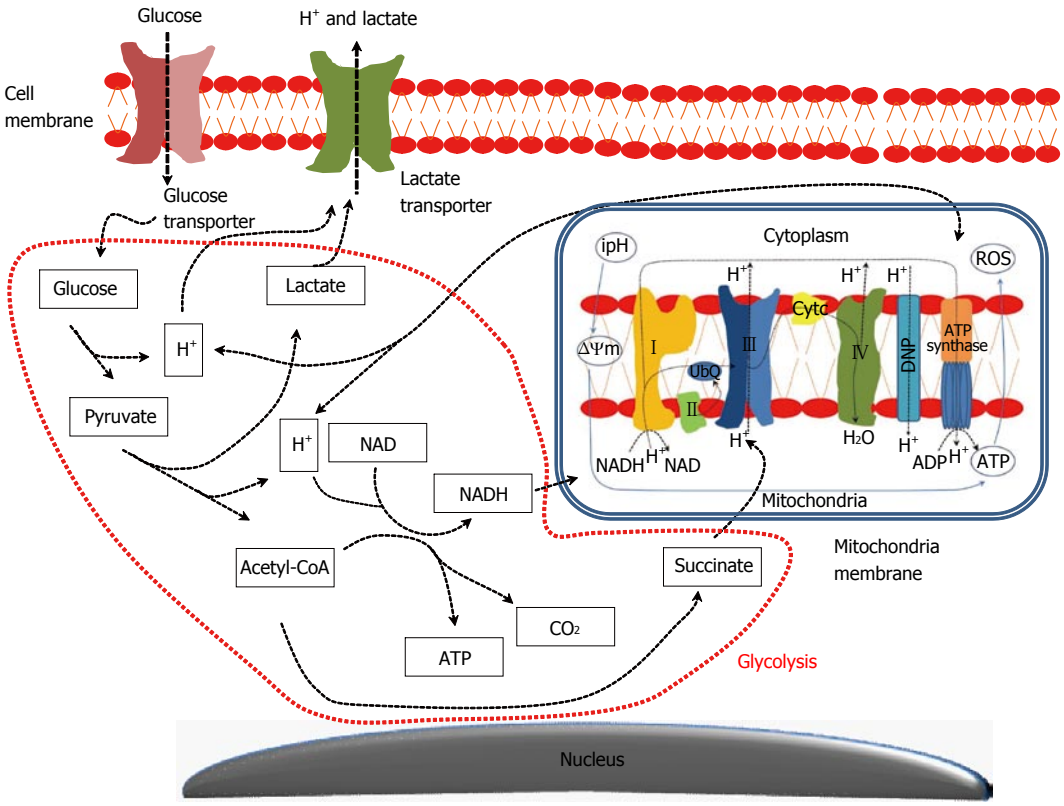

Figure 1 Main metabolic interactions lead to formation of the aerobic glycolytic metabolism in colon tumor cells. The increased biosynthetic activity of cancer cells, as related to the activation of the aerobic glycolytic metabolism or "Warburg effect", is based on the activation of glucose and lactate transporters supplying tumor cells not only with vast amounts of energy (glucose), but further reducing blockage-associated mechanisms due to glycolysis over usage. It seems that the lactate overproduction is compensated by the hyperactivation of lactate transporters allowing a rapid transport of this molecule across the plasma membrane together with H^+ atoms, which results in an intracellular alkalinization. This event hyperpolarizes the mitochondrial membrane potential ($\Delta\Psi m$) and induces a higher uptake of NADH by the first and succinate by the second mitochondrial complexes enhancing the oxidative mitochondrial phosphorylation (Krebs cycle). All together, this means that tumor cells are prone to produce higher energy amounts (ATP) than found in a normal tissue. ROS: Reactive oxygen species; CO_2: Carbon dioxide; ipH: intracellular pH. NADH: Nicotinamide adenine dinucleotide phosphate-oxidase.

ANTIDEPRESSANT FLX MODULATES OXIDATIVE STRESS

FLX was first reported by a research group from the Eli Lilly Company in 1974 as a SSRI[37]. In 1978, the United States Food and Drug Administration approved FLX for the treatment of patients with depression, anxiety and insomnia; this medication became known worldwide as Prozac[38,39]. This antidepressant exhibits higher safety and fewer side effects than other groups of antidepressants[38-41]. FLX was characterized as a lipophilic weak base, which when administered orally experiences a direct contact with epithelial cells in the intestines. In these epithelial cells, it induces an increase in serotonin (5-HT) levels by blocking L-monoamine oxidase and serotonin reuptake transporters[41-43].

On the other hand, FLX has been shown to interfere with the OS machinery in experimental models and humans[44-55]. Treatment with FLX was found to reduce malondialdehyde (MDA) and carbonyl levels in stressed rats, whilst it enhanced superoxide dismutase (SOD), catalase, glutathione S-transferase, glutathione reductase and glutathione contents[45,46]. Similar findings were reported by another research group[48]. Then, this compound showed neuroprotective effects, decreasing the translocation of p67 protein and ROS generation (by suppressing the activation of nicotinamide adenine dinucleotide phosphate-oxidase oxidase and inducible nitric oxide synthase) in rats exposed to lipopolysaccharide[47]. In depressive patients, FLX was found to decrease serum MDA, SOD and ascorbic acid levels[44].

FLX AND TUMORS

Tutton and Barkla first revealed the anticancer potential of FLX against colon tumors[56]. However, in 1992, Brandes and colleagues reported a 40% increase of the numbers of mammary fibrosarcomas among mice treated with FLX for 5 d, which was followed by findings of a 95% enhancement in breast cancer incidence after 15 wk[57]. Opposite to that, Volpe *et al*[58] showed that treating human and murine breast tumor cell lines with FLX *in vitro* did not stimulate tumor cell proliferation, DNA synthesis or colony formation. Jia *et al*[59] reported that FLX did not enhance the growth of pancreatic tumors. Moreover, this treatment was further found to reduce lymphoma growth, modulating the T-cell-mediated im-

munity reaction through a 5-HT-dependent activity[40].

In patients, FLX treatment was reported to reduce the risk of colon cancer to almost 50%[60]. Chubak *et al*[61] also observed that FLX reduced the risk of colon cancer in humans, while one meta-analysis study suggested that FLX does not act on colon cancer[62]. Studies with animal models support the idea of FLX reducing colon cancer incidence in different animal models, such as carcinogen induced preneoplastic lesions and tumors in rats and mice, and xenograft tumors in immunosuppressed rats[38,63-65]. These studies have mainly been focused on the antiproliferative effects of FLX treatment in colon tumorigenesis[38,63-65]. In cell culture models, FLX was reported to not only inhibit multidrug resistance and increase the intracellular doxorubicin concentration[66], but also to induce a further nuclear distribution of this chemotherapeutic drug[67].

FLX reduces preneoplastic lesions acting on colonic microenvironment

We have reported that FLX treatment counteracted the carcinogen-induced dysplasia in two different experimental colon cancer models[64,65]. Our first report revealed FLX as a chemopreventive compound against colonic dysplasia since treatment with FLX was started before the treatment with the carcinogen[65]. We then reported that FLX could also reduce pre-existent colon preneoplastic lesions[64]. Our findings suggested that FLX takes the carcinogen-induced preneoplastic changes under control by reducing epithelial proliferation[38,56,60,61,64,65].

Besides the fact that FLX treatment reduced dysplasia and preneoplastic angiogenesis, decreasing the epithelial and subepithelial proliferation[64,65], our unpublished dataset further suggests that by suppressing the NF-κB nuclear activity, through increased expression of cytoplasmic NF-κB-inhibitor IκB-α and IκB-β proteins, FLX reduced *c-Myc* expression and then stromal proliferation (Figures 2 and 3). As we will discuss next, FLX treatment seems to take preneoplastic angiogenesis under control by reducing the proliferation of subepithelial cells (Figure 4). Indeed, NF-κB-transcriptional activity was reported to induce the transformation of subepithelial cells from normal to reactive phenotypes, enhancing the expression of pro-inflammatory molecules and periendothelial cell numbers[68,69]. Koh *et al*[38] reported that FLX inhibited NF-κB signaling in colonic epithelial tumor cells. Inhibition of the NF-κB-transcriptional activity actually yields reduced expression of its downstream genes c-*Myc* and vascular endothelial growth fator, which blocks the proliferation of colon cancer cells[70,71].

The activity of FLX on the colonic preneoplastic microenvironment further includes the question whether this treatment could directly act upon angiogenesis-related cell phenotypes[64,65]. We have demonstrated that the anti-angiogenic potential of FLX could be related to its control over the differentiation and further transition of endothelial cells through different angiogenesis-related stem cell markers in colon preneoplastic lesions (Figure 4)[64]. This idea was abetted by the discovery of a small subset of stromal spindle cells expressing CD133 and CD34 in angiofibromas, which suggests tumors promoting subepithelial resident cells to transit towards endothelial cell phenotypes[72]. Endothelial progenitor cells were then shown to lose, in a process related to high proliferation[73], the expression of CD133 during their differentiation into vascular cells, while the expression of CD34 was increased[74-76]. Considering that CD31-positive cells have been designated as mature endothelial lineage promoting microvessels[77], vascular smooth muscle cells were found to increase the expression of CD31 during their differentiation process, whilst a simultaneous decrease of CD133 and CD34 progenitor markers was previously observed[78,79].

FLX TAKES ENERGY GENERATION UNDER CONTROL TO REDUCE MALIGNANT EXPANSION

Here, we should pull a few points together about malignancy, ROS production and energy generations, as: (1) unbalancing the machinery for energy generation induces ROS production; (2) ROS production is one of the main known events inducing DNA damage and mutation; (3) ROS generation promotes genetic mutations leading to the manifestation of preneoplastic lesions; (4) tumor cells undergo deep metabolic changes to survive and promote malignant expansion; (5) tumors enhance ROS production to promote growth through malignant molecular signaling; and (6) malignant metabolism seems to be the Achilles' heel in tumors. These few remarks give us the notion that metabolism, or energy generation, is a key for malignant transformation, tumor manifestation and growth, as well as a valuable tool for anticancer therapy[35,80-82].

As a lipophilic weak base[42], FLX quickly diffuses through multiple body-sites[83]. We have already demonstrated that FLX treatment arrested colon tumor cells within the G_0/G_1 cell-cycle phase without inducing DNA damage[64]. Then, FLX was shown to reduce ROS generation, reversing the melanoma-induced tissue oxidation in mice[50]. In brain tissue of tumor-bearing mice, FLX treatment further reduced OS, enhancing the SOD activity[49]. Actually, FLX was twice reported to stimulate Ca^{2+} flux reducing the B-cell lymphoma 2 (bcl-2) expression and mitochondrial membrane potential ($\Delta\Psi m$), which induced DNA fragmentation and apoptosis in Burkitt's lymphoma cells[52,53]. Another lipophilic weak base ([Z]-5-methyl-2-[2-(1-naphthyl) ethenyl]-4-piperidinopyridine [AU-1421]) was also reported to uncouple mitochondrial oxidative phosphorylation, dissipating the proton motive force during its energized state, which inhibited ATP synthesis[84]. It is known that lipophilic weak bases, such as FLX, reduce $\Delta\Psi m$ (or extra- and intra-mitochondrial motions of H^+ atoms generating positive charges in the mitochondrial membrane) in their energized or proton-

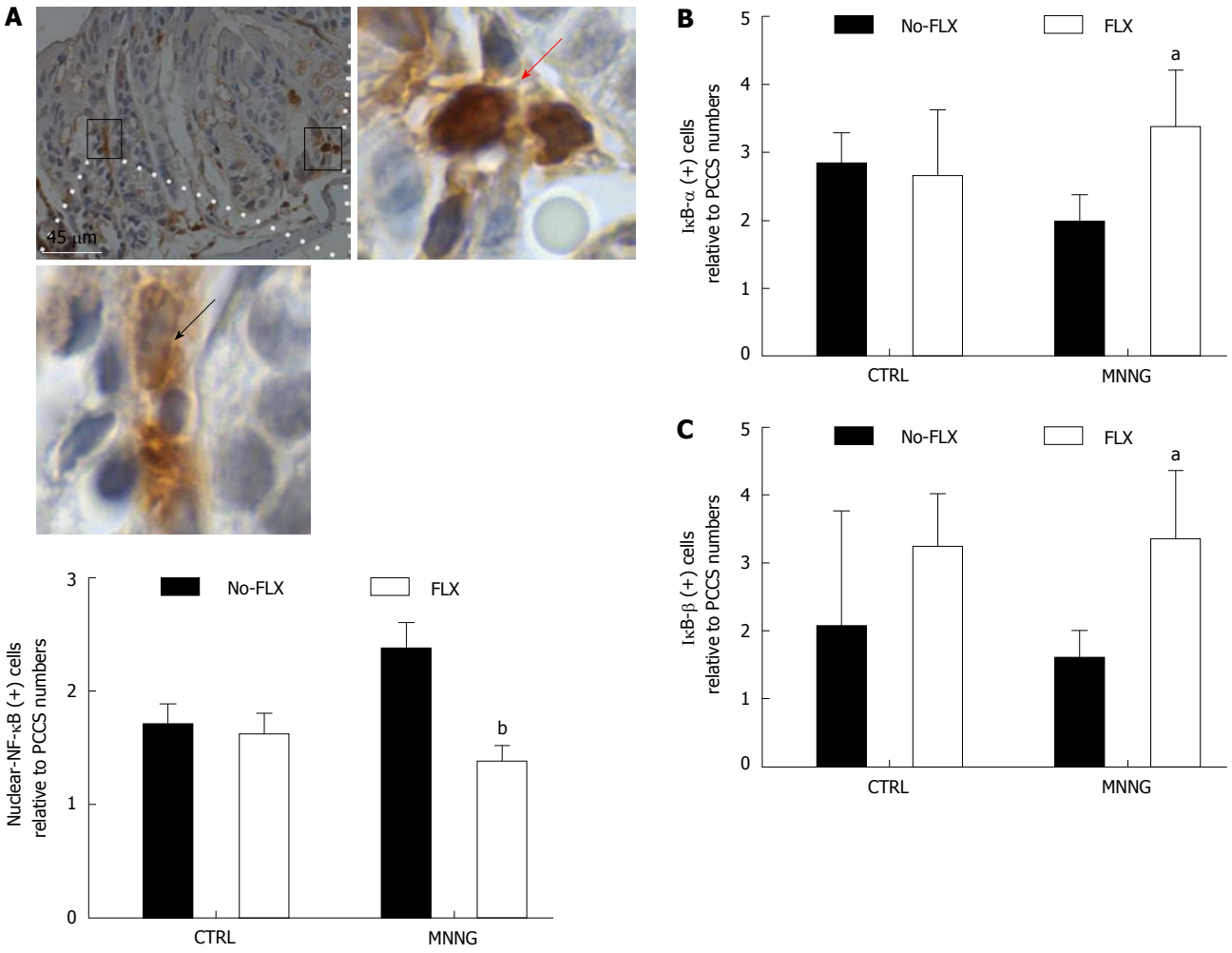

Figure 2 Fluoxetine modulates nuclear factor kappa-B nuclear activity among subepithelial colonic cells. For this figure, groups of female C57BL/6 mice (25 g) consisted of control (CTRL) animals or received methylnitronitrosoguanidine (MNNG) treatment [four successive doses of MNNG (5 mg/mL; intrarectal deposits of 100 μL) twice a week for 2 wk], FLX treatment (30 mg/kg per day; intraperitoneal, *ip*) or MNNG + FLX treatment. FLX treatment was started after 2 wk from the end of MNNG treatment, and continued for the next 4 wk. All mice were euthanized by CO_2 exposure at week 8. Individual autopsies were performed and colon tissue samples were fixed in paraformaldehyde buffer (4%; 24 h). All experimental protocols were approved by the Internal Animal Care, Ethical and Use Committee (n° 068/2012). Immunohistochemistry was performed with anti-nuclear factor kappa-light-chain-enhancer of activated B cells [nuclear factor kappa-B (NF-κB), p50; clone C-19], nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor (IκB), alpha (IκB-α; clone N-20), beta (IκB-β; clone H-4). Antibodies were acquired from Santa Cruz Biotechnology (Heidelberg, Germany). A: Representative histological image of a colonic-longitudinal section labeled with anti-NF-κB antibody, picture taken at × 400 magnification and scale bar of 45 μm inserted. A cytoplasmic anti-NF-κB antibody positively cell detected within cryptal area (inset below; × 1000 magnification of the boxed region, middle-left). Nuclear-NF-κB protein detected in stromal cells (inset right-side; × 1000 magnification of the boxed region, middle-right). Graph shows the relative number of nuclear-NF-κB positive cells within colonic subepithelial areas (PCCS; $^{b}P < 0.01$ *vs* MNNG without FLX, $n = 4$; FLX + MNNG, $n = 4$); B: Relative number of IκB-α positive cells ($^{a}P < 0.05$ *vs* MNNG without FLX, $n = 4$; FLX+MNNG, $n = 4$); and C: IκB-β positive cells within colon stromal areas ($^{a}P < 0.05$ *vs* MNNG without FLX, $n = 5$; FLX + MNNG, $n = 4$). FLX: Fluoxetine. PCCS: Pericryptal colonic stroma.

ated state, which reduces mitochondrial respiratory rate and energy generation[84-86]. FLX was also found to induce ROS generation in human ovarian cancer cell lines, which induced apoptosis through mitochondrial bcl-2-associated X protein, cytochrome c release, caspase-3 activation and p53 expression levels, whilst this treatment further reduced ΔΨm, BH3 interacting-domain death agonist and bcl-2 levels[54]. Similar findings were reported in human neuroblastomas[55].

Comparing those reports that describe how FLX modulates tumor metabolism[49,50,52-55] with others describing its activity against tumor growth[40,58,87-92], it becomes clear that FLX blocks tumor cell proliferation by impairing the malignant energy generation. The anti-tumor proliferative effects of FLX[40,56,92,93] have been related to different causes, such as delays in cell-cycle progression by inhibiting DNA synthesis and also to a possible binding directly to DNA *via* groove mode and high attraction force[58,87-90,94]. On a molecular level, FLX was shown to arrest breast tumor cells at G_0/G_1 phase by disrupting skp2-CKS1 assembly, which is required to enable cell cycle progression[91]. Recent reports have been supporting the idea of FLX acting against tumor proliferating cells by reducing *c-Myc* and cyclins (D1, D3, E, B and A), whereas cell-cycle checkpoints (p15, p16, p21, p27 and p53) were enhanced[40, 91, 92].

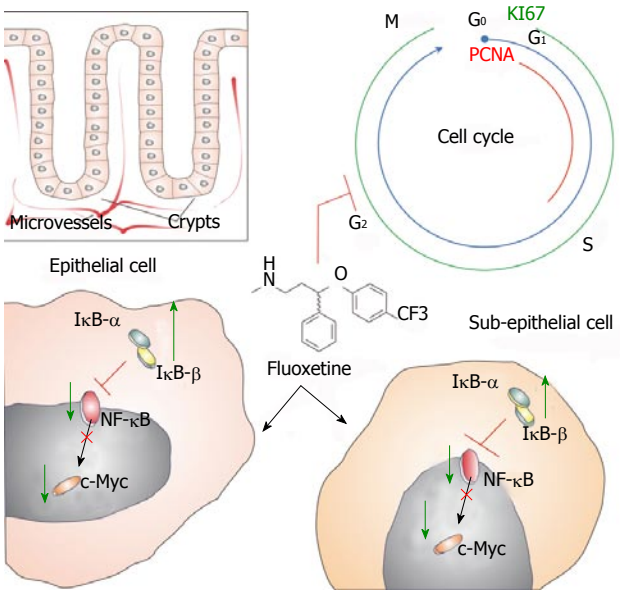

Figure 3 Schematic illustration shows fluoxetine antiproliferative activities in colon tissue. Boxed figure shows the clear division between epithelial and subepithelial colonic areas. Considering that crypts compose the colonic epithelia, it is known that microvessels surround these gland structures. Fluoxetine (chemical structure represented at the center) blocks cell-cycle (blue line and letters) in colonic tissue. We have observed that fluoxetine treatment reduced two proliferative markers, named proliferating cell nuclear antigen (PCNA, red line) and KI67 (green line). These effects of fluoxetine treatment might be related to its enhancement on IκB-α and IκB-β proteins. This could arrest nuclear factor kappa-B (NF-κB) protein in the cytoplasm, reducing its transcriptional activity which, due to its activation over c-Myc transcription factor, would decrease this protein activation and proliferation. We believe that a similar mechanism could take a place in epithelial and subepithelial cells.

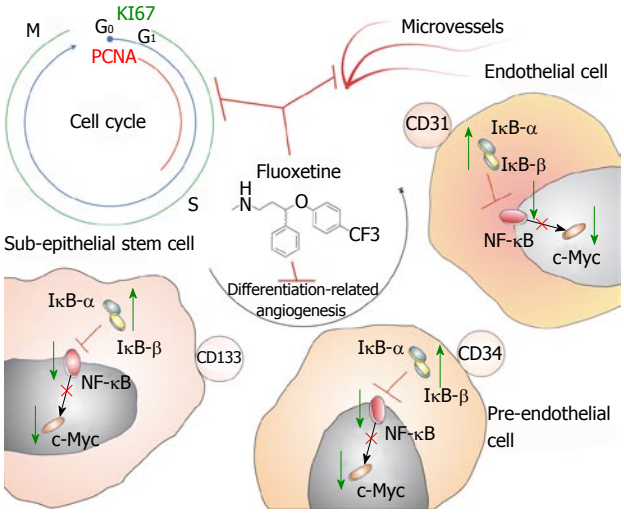

Figure 4 Schematic illustration shows fluoxetine anti-angiogenic potential in colon preneoplastic tissue. This means that by reducing proliferation of subepithelial cells, blocking their cell-cycle, fluoxetine would reduce microvessel density. This anti-angiogenic potential was observed in a direct relationship with reduced differentiation-related angiogenesis of subepithelial stem cells. This suggests that fluoxetine would reduce the differentiation of CD133 positive cells into a CD34 phenotype, which would also not differentiate in endothelial cells, as CD31. This sequence of events would mainly be associated with the control of fluoxetine treatment on nuclear factor kappa-B signaling, as reducing proliferation and preneoplastic angiogenesis. PCNA: Proliferating cell nuclear antigen.

Perspectives in FLX treatment acting against colon cancer

The application of FLX for tumor patients has so far been limited to its use as an antidepressant, but it might provide much more benefit, potentially making it an interesting co-chemotherapeutic agent. FLX treatment seems to block tumor growth by breaking the malignant metabolism down[49,52-55]. While the pieces for this puzzle are slowly being pulled together, there are already several reports which have given the ground ideas for following investigations[38-40,49,50,52-56,58,60,61,64-67,87-92,95]. Besides the specific idea of FLX acting against the tumor metabolism, there is an open question regarding the effects of FLX treatment against the "reverse Warburg effect". Pavlides *et al*[96] have suggested the idea of a reverse Warburg effect taking place in tumors; this idea argues that epithelial cancer cells induce the subepithelial cells to undergo aerobic-glycolysis and secrete lactate and pyruvate, which malignant cells would take up to enhance their tricarboxylic acid cycle, not only to generate more energy through mitochondrial phosphorylation, but further increase redox mechanisms which in turn corroborates with tumor cell survival and proliferation[30,31,82]. Schulze and colleagues have extensively reviewed this topic[30,82]. Such a mechanism would efficiently ensure enough energy production for malignant cells within the hostile tumor environment, allowing not only high proliferative rates, but the enhancement of malignant angiogenesis[97-101]. These authors have further shown that enhancing the subepithelial NF-κB signaling is closely associated with "reverse Warburg effect" in tumors[96].

Our findings, that FLX treatment reduced the nuclear detection of NF-κB protein among preneoplastic subepithelial cells (as related to reduced angiogenesis due to fewer subepithelial cellular proliferation[64,65]), lead towards the idea of FLX treatment having similar effects on subepithelial cells which surround epithelial cells in colon tumors. Figure 5 illustrates that malignant microvessels show high-cytochrome C oxidase activity in colon xenograft tumors. Moreover, our new experiments (unpublished dataset) argue that FLX treatment, in different colon tumor models, takes the malignant metabolism-related energy generation in epithelial cells under control to shrink tumors. We strongly believe that FLX counteracts aerobic glycolysis reducing the activity of lactate transporters that inhibits oxidative phosphorylation due to increased intracellular levels of lactate. This might bring down the ipH values blocking the tumor energy generation machinery. After having this hypothesis challenged in experimental models and by different research groups, we could think of clinical trials for FLX as a co-chemotherapeutic agent in colon cancer patients. Because of the low costs of FLX, this would also be transferable to developing countries with their tightly limited budget for cancer therapy.

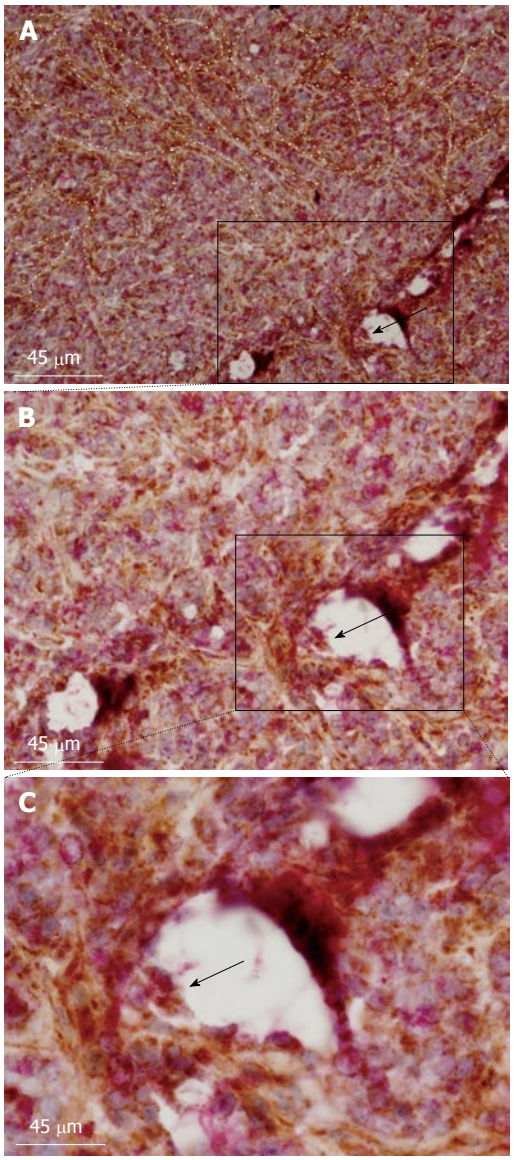

Figure 5 Tumor metabolism and malignant angiogenesis. Histopathological images show double staining between cytochrome C oxidase (COX) and anti-CD31 antibody (clone 1A10 at 1:100; Novocastra, United States). Microvessel walls are traced with sectioned white lines (horizontal view of sectioned tumor microvessels). Black arrow indicates a microvessel lumen with double-stained cells (boxed region; transversal view of a tumor microvessel). Picture was taken at × 100 magnification and 45 μm scale bars are inserted in all images. Inset (C) shows the same boxed region at × 200 magnification. Double-stained cells are pointed out by a black arrow at the microvessel wall (inset; B). Sectioned green line circulates a niche of double-stained endothelial cells at the edge of a microvessel bifurcation. To build these images, 5 wk (20 ± 2 g) nonobese diabetic, severe combined immunodeficient mice (NOD/SCID) were subcutaneously transplanted with HT29 cells (1.5 × 106 cells per mice) in agreement with the protocol approved by the Internal Animal Care, Ethical and Use Committee (n° 121/2012). All mice were acclimated for 1 wk before starting the experiment and maintained under specific pathogen-free conditions. Tumor volume was monitored throughout the whole experimental period by measures with a caliper. Mice were sacrificed under general anesthesia (1.5% Forane in 98.5% oxygen; 2l min). Tissue samples were frozen within TissueTek (Sakura, Germany) and kept at -80 ℃ for immunohistochemical analyses. Double-staining was performed according to our standard methods.

CONCLUSION

To summarize, research data concerning the activity of FLX treatment against tumor metabolism are still very limited but exciting enough to warrant new investigations. The fact that FLX was designed as an antidepressant but was further found to act against tumors already highlights that new drugs can be developed from it. Additionally, cancer therapy lacks alternative strategies to overcome chemoresistance. In many cases, chemoresistance is closely associated with tumor metabolism. It seems reasonable to suggest that treatments disrupting metabolic events, as might be possible with FLX, could effectively not only reduce chemoresistance, but also malignant angiogenesis. Whether these new perspectives for FLX treatment will be applicable for colon cancer patients are a matter of time, discussion and deeper research efforts. We strongly suggest that FLX is a promising target for further studies in cancer research.

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ORIGINAL ARTICLE

Novel blood-based microRNA biomarker panel for early diagnosis of pancreatic cancer

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Abstract

AIM: To develop a panel of blood-based diagnostic biomarkers consisting of circulating microRNAs for the detection of pancreatic cancer at an early stage.

METHODS: Blood-based circulating microRNAs were profiled by high throughput screening using microarray analysis, comparing differential expression between early stage pancreatic cancer patients (n = 8) and healthy controls (n = 11). A panel of candidate microRNAs was generated based on the microarray signature profiling, including unsupervised clustering and statistical analysis of differential expression levels, and findings from the published literature. The selected candidate microRNAs were then confirmed using TaqMan real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR) to further narrow down to a three-microRNA diagnostic panel. The three-microRNA diagnostic panel was validated with independent experimental procedures and instrumentation of RT-qPCR at an independent venue with a new cohort of cancer patients (n = 11), healthy controls (n = 11), and a group of high risk controls (n = 11). Receiver operating characteristic curve analysis was performed to assess the diagnostic capability of the three-microRNA panel.

RESULTS: In the initial high throughput screening, 1220 known human microRNAs were screened for differential expression in pancreatic cancer patients versus controls. A subset of 42 microRNAs was then generated based on this data analysis and current published literature. Eight microRNAs were selected from the list of 42 targets for confirmation study, and three-microRNAs, miR-642b, miR-885-5p, and miR-22, were confirmed to show consistent expression between microarray and RT-qPCR. These three microRNAs were then validated and evaluated as a diagnostic panel with a new cohort of patients and controls and found to yield high sensitivity (91%) and specificity (91%) with an area under the curve of 0.97 (P < 0.001). Compared to the CA19-9 marker at 73%, the three-microRNA panel has higher sensitivity although CA19-9 has higher specificity of 100%.

CONCLUSION: The identified panel of three microRNA biomarkers can potentially be used as a diagnostic tool for early stage pancreatic cancer.

Key words: MicroRNA; Diagnosis; Biomarkers; Pancreatic cancer; Blood plasma; Circulating

Core tip: This study employed high throughput screening as a screening tool to identify blood-based circulating microRNA markers for detection of early stage pancreatic cancer. Two levels of confirmation were performed to ensure the validity of the identified microRNA targets. First, a panel of potential microRNA

markers was generated and confirmed using a more specific and sensitive secondary assay, real-time quantitative reverse transcription polymerase chain reaction. Second, the confirmed panel of microRNA markers was independently validated with different experimental procedures and instruments, by independent personnel, and at a different institution, to diagnose a new cohort of patients and controls.

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INTRODUCTION

Pancreatic cancer is one of the most lethal human cancers and continues to be a major unsolved health problem[1]. It has a five-year survival rate of only 6% and is estimated to have 43920 new cases and cause 37390 deaths in 2012 in the United States, a number that has been steadily increasing for more than a decade[2,3]. Conventional treatment approaches such as surgery, radiation, chemotherapy, or a combination thereof have had little impact on the course of this aggressive cancer. Collective studies from Japan indicate that those patients who were incidentally found through other imaging modalities to have early stage carcinoma have an improved five-year survival rate of 30% for those with a 2 cm carcinoma, 57% for those with a 1 cm or less "minute" carcinoma, and 100% for patients with a ductal epithelium tumor measuring less than 1 cm[4-6]. Therefore, these studies emphasize that the hope for better control of this disease is through diagnosis at its earlier stages when surgical resection may be curative.

The current most widely used biomarkers for pancreatic cancer are CA19-9, and, to a lesser degree, carcinoembryonic antigen (CEA)[7] and genetic markers such as *K-RAS* and p53[8]. Whereas clinicians may rely on CA19-9 levels as a prognostic tool when managing patients with late stage disease, or in determining operability or monitoring patients for recurrence, these markers have generally inadequate specificity and unreliable sensitivity to pancreatic cancer and are not recommended for screening and diagnosis of early disease[9,10]. Consequently, there is an urgent, unmet need for development of valid, reliable biomarkers for early detection and monitoring of pancreatic cancer.

MicroRNA (miRNA) are small non-coding RNA about 18-24 nucleotides in size[11]. A large body of evidence indicates that miRNAs regulate gene expression at the post-translational level in almost every biological event and play important roles in tumorigenesis, cancer development, migration and metastasis[12]. The differential expression of miRNAs has been related to various cancers, and efforts have been made to profile the global miRNA expression patterns associated with these cancers[13]. Numerous investigations have evaluated the miRNA signature of pancreatic cancer, utilizing pancreatic tumor tissue and cell lines, searching for biomarkers and their association with tumorigenesis and patient survival[14-20]. However, tissue-based miRNA signature profiling is limited to the availability of tissue specimens. Therefore, it would be technologically challenging to detect cancer at its earlier stages when the tumor size is still small and proper tissue procurement may be difficult. Thus, a simple, noninvasive procedure, such as blood-based signature profiling, would be ideal for detecting pancreatic cancer at earlier stages.

Recent studies have shown that miRNAs are relatively stable and can be readily extracted and detected in bodily fluids such as blood plasma[21]. Therefore, the presence of circulating extracellular miRNAs can potentially be used as markers for cancer detection in a noninvasive way[22]. Studies investigating the potential role of circulating miRNAs as pancreatic cancer markers have shown some promise although the findings have been limited to a small number of predefined target miRNAs[23-25]. High throughput screening studies of known circulating miRNAs as biomarkers of pancreatic cancer have only recently begun to emerge, and by combining the efforts from all researchers in this area, a final panel of ideal markers can be developed to combat this dreadful disease[26-28].

This is a pilot study to employ high throughput screening microarray technology to screen for 1220 known human miRNAs (based on the miRBase version 16.0 database released in 2010)[29], comparing the differential expression signature among eight early stage pancreatic cancer patients and eleven healthy controls. The identified panel of miRNAs were subsequently confirmed, and also validated for their diagnostic potential in an independent cohort of eleven early stage pancreatic cancer patients.

MATERIALS AND METHODS

Participant population

This study was reviewed and approved by the Valley Hospital Institutional Review Board. Written informed consent was obtained from all study participants. The pancreatic cancer patient group included stage ⅡA/ⅡB patients whose stage was confirmed post-operatively by pathologists from the Valley Hospital. Eight patients diagnosed with ductal adenocarcinoma were used in the microarray screening and real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR) confirmation study. A second group of eleven ductal adenocarcinoma patients was used in the validation study. The control group for all studies was comprised of eleven healthy participants with no family history of pancreatic cancer. The high risk group included eleven healthy participants who had a strong family history of pancreatic cancer, including ten participants with at least two "first degree relatives" and one participant with two "second

Table 1 Participant demographics

	n	Median age (IQR), yr	Female gender
Microarray analysis and RT-qPCR confirmation			
Patients	8	64 (57-65)	38%
Controls	11	46 (42-49)	46%
RT-qPCR validation			
Patients	11	68 (62-79)	46%
Controls	11	46 (42-49)	46%
High risk	11	48 (46-50)	73%

IQR: Interquartile range. RT-qPCR: Real-time quantitative reverse transcription polymerase chain reaction.

degree relatives" having a pancreatic cancer diagnosis. The participant demographics are shown in Table 1.

Patients were excluded from the study if they had prior pancreatic cancer surgery, had other concomitant cancers other than non-melanoma basal cell skin cancer, or had a history of HIV infection.

Blood specimen collection and processing

Patient blood was drawn by peripheral venipuncture into BD Vacutainer® CPT™ (Cell Preparation Tubes) with Sodium Citrate (Becton Dickinson, Franklin Lakes, NJ). Blood processing was typically done within two hours of collection and performed according to the manufacturer's protocol. Harvested plasma was stored at -80 ℃.

MiRNA microarrays and analysis (performed at Ocean Ridge Biosciences, FL)

Plasma samples were processed at Ocean Ridge Biosciences (ORB, Palm Beach Gardens, FL) for analysis using custom multi-species microarrays containing 1209 probes covering 1220 human mature miRNAs present in the miRBase version 16.0 database released in 2010. The sensitivity of the microarray is such that it could detect as low as 20 amoles of synthetic miRNA being hybridized along with each sample. The microarrays were produced by Microarrays Inc. (Huntsville, Alabama), and consisted of epoxide glass substrates that have been spotted in triplicate with each probe.

Sample processing: Samples were isolated from 0.7 to 1.0 mL of plasma using TRI Reagent® BD (Molecular Research Center, Cincinnati, OH) as per manufacturer instructions. For quality control, a mixture of 10 synthetic miRNAs were added (spike-in) at a mass of 12.5 fmoles/mL of plasma to each plasma sample during isolation and one miRNA was added at 200 amoles per sample prior to labeling and hybridization. Total RNA was 3'-end labeled with Oyster-550 fluorescent dye using the Flash Tag RNA labeling Kit (Genisphere, Hatfield, PA). Labeled RNA samples were hybridized to the miRNA microarrays according to conditions recommended in the Flash Tag RNA labeling Kit manual. The microarrays were scanned on an Axon Genepix 4000B scanner, and data was extracted from images using GenePix v4.1 software.

Data pre-processing: Spot intensities were obtained for the 8816 features on each microarray by subtracting the median local background from the median local foreground for each spot. The 95th percentile of the negative control spots was also calculated for each array. The spot intensities and 95th percentile of negative controls (TPT95) were transformed by taking the Logarithm base 2 (indicated as $\log_2$) of each value. The normalization factor (N) for each microarray was determined by obtaining the 20% trimmed mean of the human probe intensities that were detected one $\log_2$ unit above TPT95 (TPT95 + 1) in all samples and with standard deviation of probe intensities among all samples less than 1.25. The $\log_2$-transformed spot intensities for all 8816 features were normalized, by subtracting N from each spot intensity, and scaled by adding the grand mean of N across all microarrays. The mean probe intensities for each of the 1209 human probes on each of the 20 arrays were then determined by averaging the triplicate spot intensities. Spots flagged as poor quality during data extraction were omitted prior to averaging. The 1209 human non-control $\log_2$-transformed, normalized, and averaged probe intensities were filtered to obtain a panel of 290 human miRNA probes showing probe intensity greater than one $\log_2$ unit above TPT95 (> TPT95 + 1) in at least 10% of the samples.

Quality control: Sensitivity of the microarray hybridization was confirmed by detection of hybridization signal for all 11 spikes that were added during isolation and labeling well above TPT95. The array also contains a set of specificity control probes complementary to three different miRNAs. Each specificity control includes a perfect match, single mismatch, double mismatch, and shuffled version of the probe. Specificity of the hybridization was confirmed by detection of hybridization signal on the microarray for the perfect match probes and not the double mismatch and shuffled version of the probes. Reproducibility of the arrays was determined by monitoring the hybridization intensity for the triplicate human spots on each array.

Differential expression analysis: For statistical analysis, samples were binned in two groups: Healthy Controls and Pancreatic Cancer. The $\log_2$-transformed and normalized spot intensities for the 290 detectable human probes were examined for differences between the groups by 1-way ANOVA using National Institute of Ageing Array Analysis software[30]. This ANOVA was conducted using the Bayesian Error Model and 50 degrees of freedom. A total of 116 probes exhibited significant differences between the healthy controls and the pancreatic cancer patients. Statistical significance was determined using the False Discovery Rate (FDR) method[31]; an FDR < 0.15 was considered significant in this study.

Hierarchical clustering of miRNA array data: Data for the 290 detectable human probes were clustered using Cluster 3.0 software[32]. Genes were median-centered prior to hierarchical clustering. Hierarchical clustering

was conducted using Centered Correlation as the similarity metric and Average Linkage as the clustering method. Intensity scale shown is arbitrary.

Confirmation with quantitative real-time RT-PCR (RT-qPCR, performed at ORB)

Total RNA was extracted from 0.9 to 2.1 mL of plasma using TRI Reagent® BD (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions with minor modifications. For down-stream quality control monitoring, a mixture of 10 synthetic miRNAs were added at a mass of 12.5 femtomoles/mL of biofluid following homogenization of the samples in Trizol-BD.

RNA isolated from plasma was diluted and used in 10 μL reverse transcription (RT) reactions using ABI (Applied Biosystems by Life Technology) miRNA-specific RT primers (Life Tech, Carlsbad, CA). For each miRNA and sample, RNA equivalent to 50 μL of biofluid was used in each RT reaction except for the probe sets for miR-642b-3p and miR-7, in which case RNA equivalent to 100 μL was used. The cDNA product was diluted 1:10 in water and 4.5 μL of the diluted product was combined in triplicate PCR-plate wells with 1 × ABI Universal PCR amplification mix and ABI miRNA-specific Taqman PCR primers in a final volume of 10 μL/well. The PCR plate was subjected to thermal cycling in an ABI StepOne Plus real-time PCR instrument. The cycling conditions were an initial incubation for 10 min at 95 ℃, followed by 40 cycles of: 15 s at 95 ℃, 1 min at 60 ℃. The Cq (Quantification Cycle according to Minimum Information for Publication of Quantitative Real-Time PCR Experiments guidelines[33], also known as threshold cycle (C_T), or number of cycles required for the well to reach a specified threshold of fluorescence intensity) was determined for each well using ABI software and a threshold setting of 0.05. In the initial pilot experiment, only miRNA with Cq value at 36 or above were used for confirmation study. Triplicate Cq values were averaged to obtain the "mean Cq value."

Independent validation RT-qPCR (performed at the Valley Hospital, NJ)

MiRNA was extracted from 2.0 to 4.0 mL of plasma using QIAamp circulating Nucleic Acid Kit (Qiagen, Germantown, MD) with QIAvac Connecting System (Qiagen), following manufacturer instructions. The concentration of extracted miRNA was determined using Agilent Small RNA kit with 2100 Bioanalyzer (Agilent, Santa Clara, CA), and was in the range of 10 to 100 ng.

RT-qPCR experiments were performed using TaqMan MiRNA Assays (Life Tech, Carlsbad, CA), for each miRNA, following manufacturer instructions. The TaqMan miRNA assays used were: hsa-miR-885-5p (ID#002296), hsa-miR-22-3p (ID#000398), hsa-miR-642b-3p (ID#462949_mat), and hsa-miR-3196 (ID#241941_mat). For RT, 1 to 10 ng RNA isolated from plasma was diluted and used in 5 μL of RT reactions with each miRNA-specific RT primers and MiRNA Reverse Transcription kit (Life Tech), without pre-amplification. The cDNA product was diluted 1:3 in water, and 5 μL of diluted cDNA was used in triplicate PCR-plate wells with miRNA-specific TaqMan PCR primer and Universal Master mix Ⅱ (Life Tech). The qPCR reaction was performed on 7500 real-time PCR system (Life Tech). The Cq value required for the well to reach a specific threshold of fluorescence intensity was determined for each well using 7500 Software (Life Tech) with threshold setting at 0.05. All Cq values were 36 or less and were included for subsequent calculation. Triplicate Cq values were averaged to obtain the "mean Cq value".

RT-qPCR and microarray data analysis using the comparative C(T) Method

The normalization control used for the RT-qPCR experiments was hsa-miR-3196. The endogenous control has been selected based on both microarray and pilot RT-qPCR experiments. MiR-3196 demonstrated relatively consistent and stable expression in microarray analysis and also in a series of pilot RT-qPCR. The use of the same extracted plasma miRNA (patients and controls, $n = 19$), with the same miRNA input, yielded consistent Cq values at 29.33 ± 0.50.

Fold change calculation was performed following the Comparative C(T) Method[34]. The "mean Cq value" of hsa-miR-3196 was subtracted from the mean Cq value for each miRNA to obtain a "normalized mean ΔCq value" for each miRNA. The "normalized mean ΔCq value" for each miRNA was then converted to $2^{-(\Delta Cq)}$. Fold change of each miRNA was determined by calculating the ratio of the mean of $2^{-(\Delta Cq)}$ of all pancreatic cancer patients to the mean of $2^{-(\Delta Cq)}$ of all healthy controls; and the mean of $2^{-(\Delta Cq)}$ of all high risk controls to the mean of $2^{-(\Delta Cq)}$ of all healthy controls. The fold change values were transformed to a $\log_2$ scale for the purpose of plotting the values on a continuum.

In order to further evaluate the RT-qPCR experimental results against those observed from the microarray analysis, microarray data for eight miRNAs (miR-642b-3p, miR-762, miR-4253, miR-885-5p, miR-18a, miR-486-5p, miR-7, and miR-22) were re-analyzed following the Comparative C(T) Method[34]. The spot intensities of all data points (in $\log_2$) were normalized by subtracting the values for each miRNA from miR-3196 (as normalization control) to generate "normalized $\log_2$ values", which were used as the equivalent of "normalized mean ΔCq value" in RT-qPCR for further calculation. The fold change values are then calculated as stated above for RT-qPCR data analysis.

CA19-9 test

The CA19-9 serum marker test was performed by the Valley Hospital Histology Laboratory following the routine diagnostic laboratory testing protocol using Tosoh A1A-360 Immunoanalyzer (Tosoh Bioscience, Inc.). CA19-9 range above 47 U/mL is considered to be a positive test result.

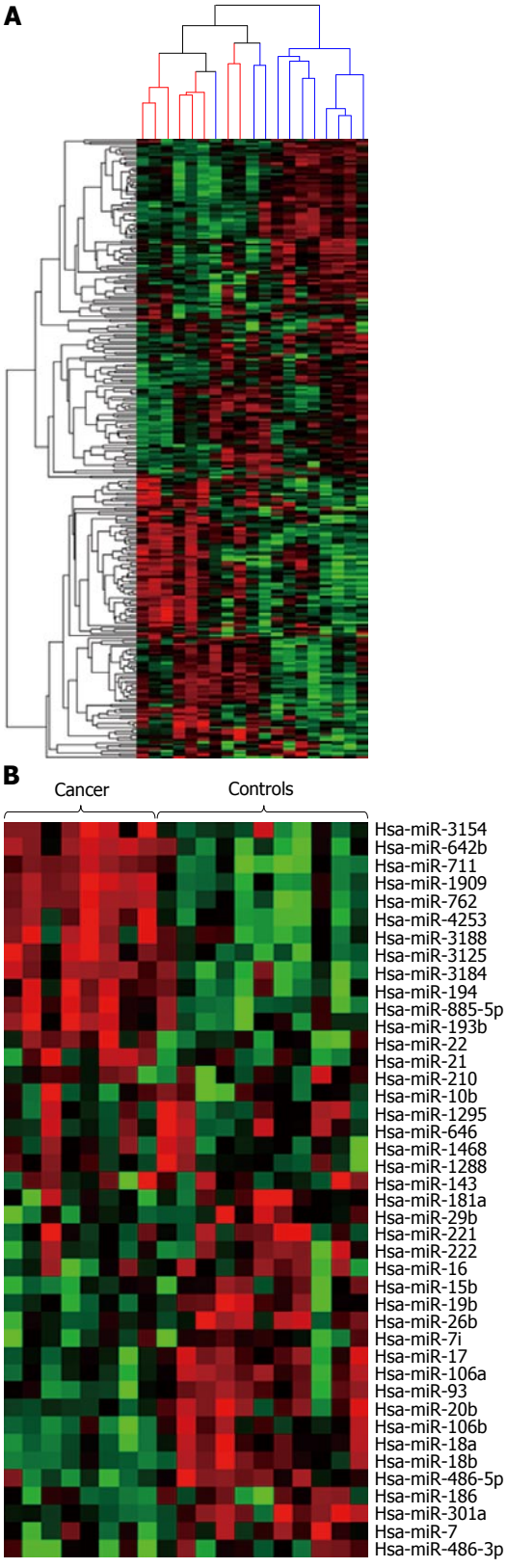

Figure 1 The microarray signature profile of circulating microRNA in pancreatic cancer patients and healthy controls. A: The unsupervised hierarchical clustering of 290 miRNAs that are differentially expressed among pancreatic cancer patients (n = 8) and healthy controls (n = 11). The dendrogram on top indicates the hierarchical clustering relationship between pancreatic cancer patients (in red) and healthy controls (in blue); B: Heat map depicting the subset of 42 miRNAs chosen for confirmation RT-qPCR study, arranged as indicated. Heat map color scale represents fold increase (red) or decrease (green) from baseline. MiRNA: MicroRNA.

Statistical analysis

The Wilcoxon-Mann-Whitney test was used to compare the fold changes observed among the patient groups for each miRNA. Receiver operating characteristic (ROC) curves were generated to evaluate the sensitivity and specificity of predicting cancer cases and control cases for each miRNA and for the combination of miRNAs. Area under the curve and their respective 95%CI were calculated for all ROC curves. P-values $\leqslant 0.05$ were considered to be statistically significant. All analyses were done using IBM-SPSS software (Version 19).

RESULTS

Circulating miRNA profiles revealed putative candidate miRNA markers

The miRNA expression signature was profiled using custom miRNA microarray chips covering 1220 human miRNAs derived from the miRBase database, version 16, released in 2010[29]. The unsupervised hierarchical clustering of the 290 miRNAs with acceptable detection intensities is shown in Figure 1A. Remarkably, as depicted in the hierarchical dendrogram, the clustering pattern clearly separates the pancreatic cancer from most healthy controls.

To compile a set of targeted miRNA markers for further investigation and confirmation, the list of 290 miRNAs was narrowed down to 31 miRNAs with significant differential expression and combined with an additional 22 miRNAs, which have been shown to be candidate biomarkers for pancreatic cancer by other investigators[14,17-20,25]. The latter criterion was added to ensure the inclusion of miRNAs which may not have exhibited significant differential expression in this experimental setting but have otherwise been shown by others to be good candidates. As shown in Table 2, among the final set of 42 miRNAs, 11 miRNAs exhibiting the most significantly different expression levels (FDR $\leqslant 0.15$) in this study (miR-194, miR-18a, miR-7, miR-26b, miR-301a, miR-106b, miR-16, miR-93, miR-106a, miR-19b, and let-7i) as well as in the previously published literature. The heat map of the 42 miRNAs in Figure 1B demonstrates a differential expression signature between pancreatic cancer patients and controls similar to the results observed from the unsupervised clustering in Figure 1A.

RT-qPCR confirmed three potential miRNA diagnostic markers

The panel of 42 miRNAs were subjected to further investigation using real time quantitative RT-PCR (RT-qPCR). In the initial pilot experiment using available miRNA TaqMan probes and testing the relative abundance of expression, 8 of the 42 miRNAs gave acceptable and most consistent signals (data not shown) and therefore were chosen for the subsequent confirmation study.

As shown in Figure 2, when comparing microarray and TaqMan RT-qPCR results side-by-side after normalizing both data sets with the same control, miR-3196,

Table 2 The 42 microRNAs for confirmation study

MiRNA	Fold change	FDR	Ref
Up-regulated in Pancreatic Cancer			
MiR-3184	4.5	0.005	
MiR-642b	3.3	0.028	
MiR-1909	3.0	0.030	
MiR-3154	3.5	0.064	
MiR-711	3.1	0.064	
MiR-3125	5.2	0.069	
MiR-4253	2.8	0.082	
MiR-762	2.4	0.082	
MiR-885-5p	3.0	0.103	
MiR-3188	2.4	0.154	
MiR-194	2.1	0.154	[19]
MiR-193b	2.4	0.155	
MiR-22	1.4	0.706	[19,20]
Up-regulated in Healthy Controls			
MiR-486-5p	3.0	0.064	
MiR-18b	2.9	0.064	
MiR-1288	2.6	0.064	
MiR-486-3p	2.3	0.082	
MiR-18a	2.7	0.082	[19,25]
MiR-7	2.5	0.091	[19]
MiR-26b	3.9	0.112	[19]
MiR-646	2.5	0.131	
MiR-1295	2.4	0.131	
MiR-301a	2.4	0.131	[18]
MiR-106b	2.3	0.151	[19]
Let-7i	4.0	0.154	[20]
MiR-16	3.7	0.154	[17,19]
MiR-20b	2.9	0.154	
MiR-93	2.6	0.154	[19]
MiR-106a	2.6	0.154	[17,19]
MiR-17	2.5	0.154	
MiR-19b	2.5	0.154	[19]
MiR-1468	2.2	0.154	
MiR-29b	2.1	0.171	[20]
MiR-15b	2.3	0.172	[19]
MiR-186	1.8	0.196	[19]
MiR-10b	1.9	0.250	[19]
MiR-143	2.3	0.323	[19,20]
MiR-181a	1.7	0.361	[14,19]
MiR-222	1.6	0.546	[14,17-19]
MiR-221	1.6	0.546	[14,18,19]
MiR-210	1.2	0.813	[17,19]
MiR-21	1.4	0.866	[14,18,19]

The 42 final target list based on the 290 miRNAs identified by miRNA microarray. The fold change shown is calculated as the ratio of pancreatic cancers over healthy controls (under "Up-regulated in Pancreatic Cancer") or healthy controls over pancreatic cancers (under "Up-regulated in Healthy Controls"). MiRNA: MicroRNA; FDR: False discovery rate.

the relative expression level of each miRNA varied due to the differing nature of the experimental technology. However, three of the eight miRNAs, miR-642b-3p, miR-885-5p, and miR-22-3p, demonstrate consistent outcomes across the two methodologies with higher expression seen in pancreatic cancer patients than in healthy controls. Therefore, they were chosen for further validation study as a potential diagnostic panel.

The validation study was performed on a new independent cohort of 11 pancreatic cancer patients, 11 healthy controls and 11 high risk controls. As shown in Figure 3A, the three miRNAs, plotted using the raw data

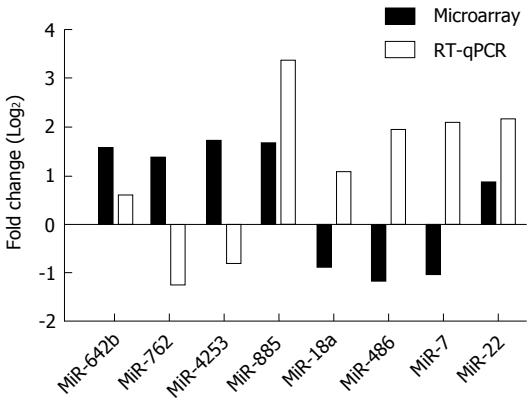

Figure 2 Confirmation study of the expression profile of eight microRNAs. Microarray (solid bar) and RT-qPCR (open bar) data sets were analyzed based on the "Comparative C(T) Method", normalized to miR-3196, and calculated as the ratio of mean fold change of pancreatic cancer patients (n = 8) to healthy controls (n = 11). MiRNA: MicroRNA.

of "normalized mean ΔCq value", differentiate pancreatic cancer patients from both the healthy and high risk controls. Likewise, as shown in Figure 3B, when determining the relative fold expression change by calculating the ratio of pancreatic cancer to healthy controls, expression of the three miRNAs is clearly up regulated in pancreatic cancer patients. For the high risk controls, on the other hand, the expression level is either comparable or lower when compared to the healthy controls. Furthermore, the relative expression levels of all three miRNAs are remarkably consistent in direction and magnitude between confirmation RT-qPCR (Figure 2) and validation RT-qPCR (Figure 3B), despite the use of different patient samples and independent experimental procedures. This lends support to the suggestion that they could play potential roles as diagnostic biomarkers for pancreatic cancer.

Assessing diagnostic potential of the three-miRNA panel

To assess the potential use of the three miRNA as a diagnostic panel for pancreatic cancer, ROC analysis was performed on the validation data set for each of the individual miRNAs and the combination of the three. As summarized in Table 3 and shown in Figure 4B and 4C, miR-885-5p and miR-22-3p each demonstrated high sensitivity of 82% for cancer case identification and relatively high specificity of 73% and 82%, respectively, for identifying healthy controls. For miR-642b-3p (Figure 4A), a high sensitivity of 82% for identifying cancer cases was demonstrated, but a lower specificity for identifying healthy controls of 55% was observed. When using the optimal cut-point, as shown in Figure 4D, the composite of all three miRNAs yielded both a sensitivity and specificity of 91%.

Given that the CA19-9 serum marker is the only marker currently available for routine diagnostic testing, we sought to compare CA19-9 results with the three-miRNA panel for predicting pancreatic cancer. The CA19-9 test has been performed on our cancer patient

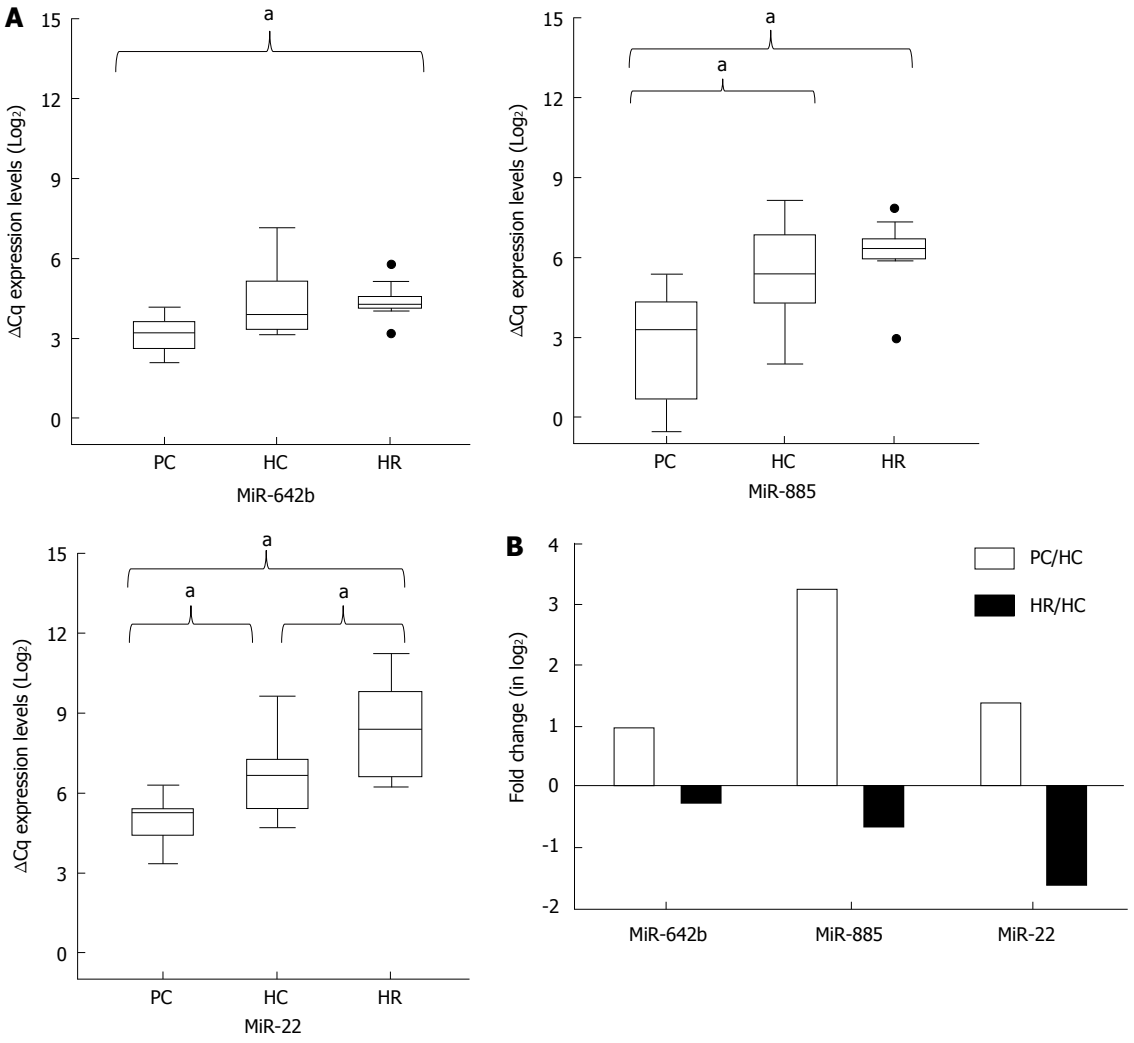

Figure 3 Validation of three-microRNA panel using quantitative real-time reverse transcription polymerase chain reaction. A: Box plot of relative expression of three miRNAs, based on normalized mean ΔCq values [mean quantification cycle (Cq) normalized to miR-3196] of pancreatic cancer patients (PC, *n* = 11), healthy controls (HC, *n* = 11), and high risk controls (HR, *n* = 11). The whiskers extend to the observations which are no more than 1.5 times the length of the box (interquartile range) away from the box. More extreme observations are considered outliers and are indicated as dots. "a" indicates *P* value ≤ 0.017 (0.05/3, a Bonferroni-adjusted α-level based on the 3 multiple comparisons performed among patient groups); B: Fold change expression levels of three miRNAs calculated as the ratio of mean fold change of PC/HC and HR/HC. MiRNA: MicroRNA.

Table 3 Receiver operating characteristic curve analysis of the 3 microRNAs validated by quantitative real-time reverse transcription polymerase chain reaction for diagnosing pancreatic cancer

MiRNA	Sensitivity	Specificity	AUC (95%CI)	*P* value
MiR-885-5p	82%	73%	0.84 (0.68-1.00)	0.006
MiR-22-3p	82%	82%	0.86 (0.70-1.00)	0.004
MiR-642b-3p	82%	55%	0.79 (0.59-0.98)	0.02
Composite of 3 miRNAs	91%	91%	0.97 (0.90-1.00)	< 0.001

AUC: Area under the curve; MiRNA: MicroRNA.

sample (*n* = 11) and non-cancer patient samples (healthy and high-risk controls, *n* = 22), and the sensitivity was observed to be 73% (8 out of 11 patients), and the specificity was observed to be 100% (all controls were below the reference point).

DISCUSSION

Early cancer detection remains a major challenge in pancreatic cancer but holds promise of resulting in a more favorable disease outcome. In light of the fact that current research progress into early detection of pancreatic cancer has resulted in limited actual clinical applications using various biomarkers such as tissue nucleic acid, proteins, tumor cells, and plasma proteins, we elected to focus on a relatively new source of potential biomarker, blood-based circulating miRNA. Given this test is blood-based, it would be noninvasive and ideal for diagnosing asymptomatic cancer. We performed an array-based high throughput screening process for all known human miRNA species (released by miRBase in 2010). We employed two levels of confirmation with RT-qPCR, using two independent samples of pancreatic cancer patients studied under two different sets of experimental conditions. We have

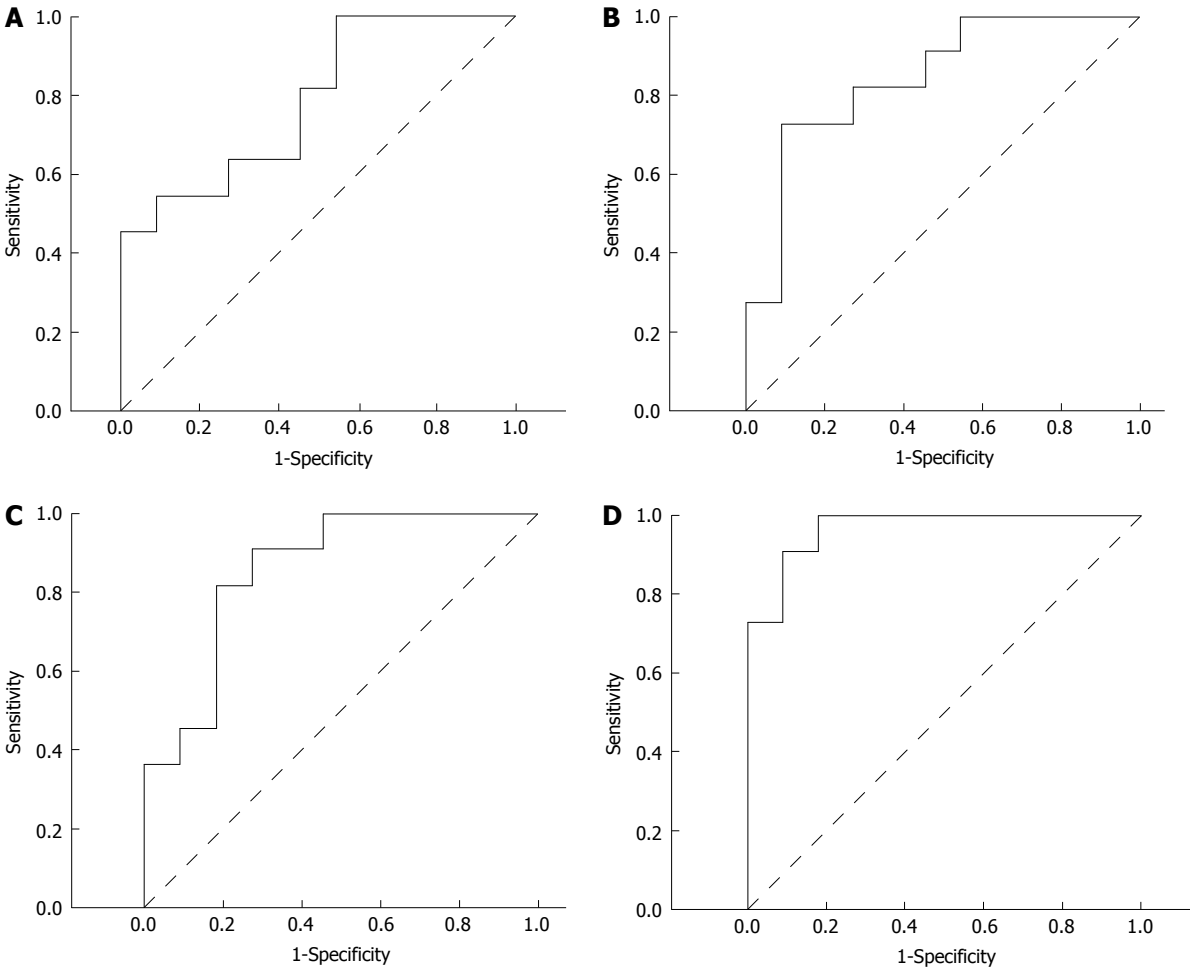

Figure 4 Receiver operating characteristic curve analysis of three microRNAs. Validation study with 11 stage ⅡA/ⅡB pancreatic cancer patients and 11 healthy controls. A: Receiver operating characteristic (ROC) curve for the miR-642b-3p data alone [area under the curve (AUC) = 0.79]; B: ROC curve for the miR-885-5p data alone (AUC = 0.84); C: ROC curve for the miR-22-3p data alone (AUC = 0.86); D: ROC curve for all three miRNAs (miR-642b-3p, -885-5p, 22-3p) as a composite panel (AUC = 0.97). MiRNA: MicroRNA.

identified and validated a panel of three miRNAs (miR-642b-3p, miR-885-5p and miR-22-3p) with high combined sensitivity of 91% and specificity of 91%.

Three prior studies have performed screening of circulating blood miRNAs for pancreatic cancer. Ali *et al*[28] profiled plasma miRNAs based on a pooled plasma specimen from 50 newly diagnosed pancreatic cancer patients (without specifying cancer staging). They identified miR-21 to be significantly higher and the expression of the let-7 family (especially let-7d) and miR-146a to be significantly lower in cancer. Liu *et al*[26] used Illumina Sequencing by Synthesis (SBS) technology and identified seven miRNAs (miR-20a, miR-21, miR-24, miR-25, miR-99a, miR-185, miR-191) as a potential panel of biomarkers, with high sensitivity of 83.6%. However, surprisingly, the SBS technology employed did not identify any new, as yet unknown miRNA markers, considering that more mature human miRNA continue to be identified and updated yearly (a total of 2578 mature human miRNA are included in miRBase version 20 issued in 2013)[29]. Also, Liu *et al*[26] screened for pancreatic cancer patients from all stages (13.2% stage Ⅰ, 24.4% stage Ⅱ, 22.8% stage Ⅲ, 33.5% stage Ⅳ, and 6.1% stage unknown). Therefore, it is unclear whether the seven-miRNA panel should be used as pan-pancreatic cancer prognostic markers or as early stage cancer screening markers, considering the gene expression profile can change dramatically from early to late stages[35,36]. Carlsen *et al*[27] used the same strategy as presented in our study and identified circulating miR-375 as the sole potential marker although with relatively low accuracy (70%), and it did not outperform CA19-9 as a diagnostic marker. However, Carlsen *et al*[27] used chronic pancreatitis patients as controls instead of normal healthy donors. It is uncertain why chronic pancreatitis patients were used as controls considering only a small population (8%) of pancreatic cancer patients have concomitant chronic pancreatitis[37]. There is a weak link between pancreatitis and pancreatic cancer[38], and only 4% of patients within 20 years of chronic pancreatitis diagnosis have developed into pancreatic cancer[39].

The research presented here focuses solely on stage Ⅱ pancreatic ductal adenocarcinoma patients, of which the gene expression profile should resemble early stage pancreatic cancer more closely than advanced stage cancer. The validation experiment was done by comparing pancreatic cancer patients to healthy controls, and in par-

ticular to high risk controls who might have inherited genetic susceptibility[40]. Our three-miRNA panel can differentiate pancreatic cancer patients from healthy controls with high sensitivity and specificity. Nevertheless, the 11 miRNAs, as presented by the three prior studies mentioned above, were also found in our 290-miRNA panel to have acceptable detection intensities (presented in Figure 1A). It would be of great interest to conduct a future larger scale clinical trial comparing the three-miRNA panel identified here to the eleven-miRNAs identified previously to see how they fare in predicting pancreatic cancer, or if the combination of all 14 miRNAs could be developed into a diagnostic test for cancer.

Of the three miRNAs identified in this study, two miRNAs (miR-642b-3p and miR-885-5p) were shown to be significantly up-regulated in cancer patients by our screening process while the third miRNA (miR-22-3p) was shown to be up-regulated in cancer patients in the literature[19,20]. It should be noted that miR-642b is a relatively novel miRNA marker with no prior publication about its potential functional role or utility as a marker. However, miR-885-5p has been shown to be a potential serum marker for liver pathologies, including hepatocellular carcinoma, liver cirrhosis, and chronic hepatitis B[41]. Functionally, miR-885-5p is found to be located in the 3p25.3 genomic region and is known to have a tumor suppressive function by triggering cell cycle arrest and senescence and/or apoptosis[42]. MiR-885-5p activates the p53 pathway, causes down-regulation of cyclin-dependent kinase and mini-chromosome maintenance protein, and suppresses matrix metallopeptidase 9 expression and Caspase genes[42-44]. MiR-22, on the other hand, is one of the most common miRNAs in the colorectal cancer transcriptome and has been studied for its critical role in breast cancer and bone metastasis[45,46]. It is known for directly targeting the estrogen receptor α mRNA[47] and is proposed to be a putative tumor suppressor by repressing the EVI1 oncogene expression[48]. It inhibits cell cycle progression by repressing Max and ErbB3 expression post-transcriptionally, mediates the effects of the tumor-suppressor p53, and suppresses interferon gene expression by blocking interferon regulatory factor-5[49-51]. MiR-22 has been proposed as a potential serum marker for non-small cell lung cancer[52], esophageal squamous cell carcinoma[53], and nasopharyngeal carcinoma[54].

Our experimental strategy in this study focused on using hybridization-based microarray technology as the means to screen thousands of genes simultaneously, but it is also severely limited due to its issues with reproducibility and its tendency to produce a high rate of false positive and false negative results. This restricts its potential use as a reliable diagnostic tool for cancer. RT-qPCR, on the other hand, is based on sequence-specific amplification, which is highly specific and sensitive for individual testing targets. It has been developed for use in diagnostic/prognostic tests such as the Oncotype DX test for breast cancer and the Cervista® HPV HR assay. Furthermore, our approach using two layers of confirmation with RT-qPCR, utilizing different sets of patients, independent experimental procedures and instrumentation, still showed a remarkable consistency that suggests the potential future application of RT-qPCR-based diagnostic tests using circulating miRNA markers.

Although CA19-9 is not considered an ideal biomarker for the early diagnosis of pancreatic cancer, data from this study demonstrated a relatively high sensitivity of 73% and specificity of 100%. It should be noted, however, that all patients recruited for this study were "confirmed cancer cases" by pre-surgery imaging [magnetic resonance imaging (MRI) and computed tomography (CT)] and post-surgical examination by pathologists. Therefore, even with 100% confirmed cancer cases, CA19-9 has only a 73% sensitivity to detect them. The three-miRNA panel, on the other hand, exhibited a 91% sensitivity. It would be of great interest to test the three-miRNA panel, alongside CA19-9, in a future large scale clinical trial of suspected cancer cases to see how the two-marker system fares when they are compared to one another or when they work together.

In summary, we have identified three blood-based circulating miRNA targets, miR-642b-3p, miR-885-5p and miR-22-3p, which, when combined, provided a high level of diagnostic accuracy for early stage pancreatic cancer. Our plan is to study an expanded sample of patients to further develop and refine the diagnostic miRNA panel based on RT-qPCR. This new panel may work alone or in conjunction with other known immunoassays, such as CA19-9 and CEA, as a diagnostic test for early stage pancreatic cancer. We envision that the future miRNA biomarker panel can immediately apply to the category of patients at high risk for pancreatic cancer before more expensive and invasive modalities like CT, MRI, endoscopic ultrasound and endoscopic retrograde cholangiopancreatography are used. A similar strategy can also be utilized to identify miRNA panels for other cancer types where early detection is crucial for a favorable disease outcome.

ACKNOWLEDGMENTS

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COMMENTS

Background

Pancreatic cancer is one of the most lethal human cancers with a mere 6% 5-year survival rate. Studies have shown that early detection is the best option available for controlling this disease. The goal of this study, therefore, is to explore and compile a diagnostic biomarker panel, based on microRNA (miRNA) in the circulating blood, for detection of pancreatic cancer at earlier stages.

Research frontiers

A diagnostic panel of biomarkers for cancer is highly desirable because it would help to fight cancer at the earliest possible stage when the disease is still curable. However, for the past fifteen to twenty years, despite numerous studies and publications, this goal remains elusive with only scanty numbers of risk assessment and prognostic panels eventually developing into clinical tests. One of the major problems is study reproducibility. The results of one study cannot be readily reproduced by another study. In addition, gene expression profiles can vary greatly among the different stages for each type of cancer, as well as between blood-based markers and tissue-specific markers. Therefore, it is critical for each investigator to be clear on their general experimental strategy which can, in turn, address issues pertaining to the development of future clinical tests.

Innovations and breakthroughs

The current study focuses on identifying a panel of blood-based biomarkers for pancreatic cancer. If it is effective, pancreatic cancer detection can be performed by a simple non-invasive blood draw instead of an invasive procedure. The approach used in this study is innovative due to the fact that, in addition to the general strategy of microarray screening followed by polymerase chain reaction-based confirmation, the authors employed a second layer of validation experiments, using different experimental procedures, instrumentation, and lab personnel at an independent location. The results of the two experimental strategies are remarkably similar even with a new cohort of patient specimens, suggesting high validity of this diagnostic panel. Furthermore, the authors have included a group of high risk individuals as controls. High risk controls are subjects with a strong family history of pancreatic cancer (at least two first degree relatives with the disease). Therefore, they have inherited genetic susceptibility to developing pancreatic cancer and hence are genotypically closer to individuals with disease than to normal healthy controls. Remarkably, the three-miRNA panel identified in this study can differentiate pancreatic cancer patients from both normal and high risk controls, demonstrating its high sensitivity and specificity for pancreatic cancer.

Applications

The identified panel of three miRNAs can potentially be used as a diagnostic detection set for early stage pancreatic cancer.

Terminology

MiRNA are small non-coding RNA approximately 18-24 nucleotides in size. The abnormal expression of miRNA found in patient blood is known to be associated with cancer progression. Hence, the utilization of miRNA biomarkers was proposed as a way to potentially detect pancreatic cancer.

Peer review

This is a well written paper examining a potentially useful means of screening for pancreatic carcinoma. The authors would recommend publication of the manuscript.

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16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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REVIEW

Thrombocytosis as a prognostic marker in gastrointestinal cancers

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Abstract

Thrombocytosis is an adverse prognostic factor in many types of cancer. These include breast cancer, ovarian and other gynecologic cancers, renal cell carcinoma and lung cancers. In gastrointestinal cancers of various locations and histologic types, thrombocytosis has been reported in general to be associated with adverse clinical outcomes. Platelet count measurement is well standardized and available in every clinical laboratory, making its use as a prognostic marker practical. This paper will discuss the data on the prognostic value of thrombocytosis in gastrointestinal cancers as well as pathogenic aspects of the association that strengthen the case for its use in clinical prognostication.

Key words: Thrombocytosis; Platelets; Cancer; Gastrointestinal; Prognosis

Core tip: Thrombocytosis arises as a prognostic factor in various cancers, although it is not clear whether there is a pathogenic contribution or thrombocytosis merely reflects a pro-carcinogenic inflammatory milieu. This paper discusses the utility of thrombocytosis as a prognostic factor in gastrointestinal cancers.

Voutsadakis IA. Thrombocytosis as a prognostic marker in gastrointestinal cancers. *World J Gastrointest Oncol* 2014; 6(2): 34-40 Available from: URL: http://www.wjgnet.com/1948-5204/full/v6/i2/34.htm DOI: http://dx.doi.org/10.4251/wjgo.v6.i2.34

INTRODUCTION

Platelets play an important role in hemostasis and vascular integrity. They have a unique mechanism of derivation as fragments from the cytoplasm of bone marrow megakaryocytes in a process called thrombopoiesis[1]. The cytokine thrombopoietin stimulates platelet production through ligation of its cognate surface receptor c-Mpl. Other signals also contribute to thrombopoiesis including SDF1 (stem cell derived factor 1, also called CXCL12) ligating receptor CXCR4, integrins and PF4 (platelet factor 4). Support is lent to megakaryocytes by the bone marrow microenvironment in the form of both soluble factors and of direct cell-cell interactions with specialized resident stromal cells[2]. Platelets are derived from proplatelets which represent long protrusions of the mature magakaryocyte cytoplasm[3]. Abnormalities in platelet number, either increase (thrombocytosis) or decrease (thrombocytopenia) accompany diverse pathologic conditions and may aid in their diagnosis[4]. An elevated platelet count has various causes and is either primary due to essential thrombocytosis or other myeloproliferative disorders or secondary to malignancy, infection, chronic inflammation, trauma or surgery, iron deficiency and splenectomy. The common denominator of most of these secondary conditions is inflammation[5]. Inflammatory cytokines stimulate the process of platelet production by megakaryocytes in the bone marrow. Cancer is a pathology that is often associated with thrombocytosis. This relates to the cytokine milieu of several malignancies that stimulates thrombopoiesis. Possibly due to this fact of association with a particular cytokines setting, thrombocytosis has been found to be an adverse prognostic

factor in many common malignancies. Thrombocytosis appears to be a universal marker of adverse outcomes in cancer. Its association with worse oncologic outcomes has been reported in early and advanced breast cancer[6,7], ovarian cancer[8,9], genitourinary cancers[10,11] and several other types[12,13].

PATHOGENESIS OF THROMBOCYTOSIS IN CANCER

A recent publication has shed some light to the pathogenesis of thrombocytosis in cancer[8] and confirmed previous reports on the role of cytokines and in particular of IL-6[14]. In ovarian cancer patients, thrombocytosis was significantly correlated with plasma levels of IL-6[8]. In mouse models bearing human ovarian cancer, human IL-6 stimulates hepatocytes through the IL-6 receptor to trigger thrombopoietin production. Thus a proposed model stipulates that ovarian cancer tumor cells produce IL-6 which then stimulates hepatic thrombopoietin production. Thrombopoietin increases thrombopoiesis through stimulation of megakaryocyte progenitors in the bone marrow[8]. In other cancers IL-6 may also play a similar role in favoring thrombocytosis and increased serum levels or tumor positivity by immuno-histochemistry have been detected in a variety of types, such as renal, prostate and breast carcinomas[15-17]. In malignant mesothelioma levels of serum IL-6 correlate with thrombocytosis[18]. IL-6 is produced locally in the tumor environment because pleural effusion levels were much higher than in serum. Interestingly in that case IL-6 may not be derived directly by mesothelioma tumor cells but by attracted immune cells because it was found that patients with tuberculous effusions had even higher levels of IL-6[18]. Specifically in gastrointestinal carcinomas, IL-6 is reported to be higher in patients with gastric and colorectal carcinoma compared to controls[19,20]. Except for the indirect effect through platelets, IL-6 has a role directly in gut carcinogenesis and possibly to chemotherapy response[21,22]. Nevertheless, IL-6 levels do not always correlate with thrombocytosis and other factors produced in bowel inflammatory microenvironment must play a role in its induction[23]. Tumor infiltrating lymphocytes and macrophages are present in various degrees in cancer sites and their role in both promoting and suppressing the tumor development is described[24]. Conditions in tumor micro-environment, such as hypoxia, affect the function of infiltrating immune cells and shape the panel of cytokines produced by them, which in their turn influence tumor cells[25]. In view of this discussion, platelet effects must be considered as constituting only part of the inflammatory process in cancer micro-environment and results of platelets influences should be interpreted with this larger perspective in mind.

The mechanistic basis of platelets contribution to carcinogenesis is a subject of investigation[26]. Circulating tumor cells may use platelets as a protective shield from the attack of the immune system and as facilitators for attachment to endothelial cells at metastatic sites. Platelets have also roles in carcinogenesis directly related to their normal function in promotion of vascular integrity[27]. Newly formed tumor vasculature lack the normal architecture and robustness of local resident vasculature and platelets have been shown to be indispensable for preventing hemorrhage in tumor beds[28]. Both alpha and dense granules of platelets carry bioactive molecules and growth factors. These include vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), platelet derived growth factor (PDGF), hepatocyte growth factor (HGF), insulin-like growth factor (IGF), transforming growth factor β (TGFβ), interleukin 1β (IL-1β), IL-8, CXC motif containing ligand 12 (CXCL12), sphingosine 1-phosphate (S1P) and lysophosphatidic acid[29,30]. Each of these molecules may actively facilitate metastatic progression. An example is platelet-derived TGFβ which promotes an EMT (epithelial to mesenchymal transition) program in cancer cells through transcription factors Smad and NF-κB signaling[31]. EMT constitutes a program endowing epithelial cells with a mesenchymal phenotype that promotes mobility and metastasis while protecting them from anoikis (Apoptosis due to lack of adhesion)[32]. Platelet-derived TGFβ may also contribute to tumor immune evasion[33]. There exist quantitative differences in platelet cargo of bioactive factors and platelets from patients with cancer have a higher VEGF level than platelets from individuals without cancer[34]. As a result platelet counts may more accurately account for VEGF concentrations in the tumor and metastases sites environment where they are activated. Interestingly IL-6 signaling through the STAT3 (signal transducer and activator of transcription 3) is able to induce VEGF receptor VEGFR2 in colorectal cancer cells[35] and thus to complete a pro-carcinogenic loop in cancer cells that includes IL-6, platelets and VEGF.

THROMBOCYTOSIS IN ESOPHAGEAL CANCER

In 293 patients with esophageal squamous cell carcinoma, thrombocytosis, defined as platelets more than 293 × 10^9/L, which was the mean plus one standard deviation of a healthy control group, was present in 21% of patients and was not correlated with patients age and gender[36]. In contrast, it was a significant independent prognostic factor for overall survival[36]. This association was statistically significant for patients with stage Ⅲ and Ⅳ but not for stage Ⅰ and Ⅱ disease. In multivariate analysis, thrombocytosis, together with higher T stage, tumor size and nodal involvement, predicted for worse survival.

In another study which included mainly patients with squamous carcinomas but also a minority (7%) with esophageal adenocarcinomas, thrombocytosis, defined this time as platelets more than 400 × 10^9/L, was present in 4% of patients and it was not associated with age, gender, location along the esophagus, degree of differentiation, lymphovascular or perineural invasion or node

involvement[37]. It was observed more often in patients with adenocarcinoma and correlated with tumor size. Although this report did not study thrombocytosis as it pertains to prognosis, either overall or progression free survival, it did confirm the finding of the previous study regarding its lack of association with other possible prognostic factors.

THROMBOCYTOSIS IN GASTRIC CANCER

In a very large series of 1593 gastric adenocarcinoma patients, 6.4% had thrombocytosis (defined as platelets more than 400 × 10^9/L in this study)[38]. All patients had undergone gastrectomy with negative margins and extensive D2 lymph node dissection. Thrombocytosis was associated with higher T stage, node positivity and a worse survival. Despite that, in multivariate analysis, the prognostic value of thrombocytosis for long term survival was lost while T stage and node positivity remained statistically significant predictors of long term survival in these patients. Thrombocytosis was a strong predictor of overall recurrence and specifically of hematogenous metastasis but not of locoregional recurrence or peritoneal seeding[38]. These predictive values were retained even in multivariate analysis in this instance.

In another series of 369 gastric cancer patients, thrombocytosis was present in 11.4% and was associated with worse 1 year and 3 year survival[39]. The 1 year survival of patients with thrombocytosis was 72.9% while of those without thrombocytosis was 85.7%. The 3 year survival of patients with thrombocytosis was 23.4% while of those without thrombocytosis was 52.4%. Thrombocytosis was positively correlated with depth of tumor invasion and lymph node involvement[39].

In a smaller series of 98 patients operated for gastric carcinoma, pre-operative thrombocytosis was present in 21% and was associated with a statistically significant worse overall survival[40]. The 5 year survival of patients with thrombocytosis was 9.5% and of patients without thrombocytosis was 31.2% in this series. Interestingly the pro-angiogenic enzyme thymidine phosphorylase/platelet-derived endothelial cell growth factor expression was associated with thrombocytosis and both were independent predictors of survival in multivariate analysis[40]. Finally, a study of 181 gastric cancer patients investigated platelet number and serum VEGF level as prognostic factors and failed to correlate either with overall or progression-free survival. In contrast the ratio of VEGF to platelet number was significantly associated with progression-free survival in multivariate analysis[41]. This may relate to the pathophysiologic importance of activated platelet derived VEGF in promoting the neoplastic process.

THROMBOCYTOSIS IN PANCREATIC CANCER

Pre-operative thrombocytosis was investigated as a prognostic factor in 109 patients with pancreatic adenocarcinoma that were surgically resected[42]. It was found to be significantly associated with reduced overall survival. Significance was confirmed in a multivariate regression analysis. Disease-free survival was also worse with thrombocytosis in a series of patients with operable pancreatic cancer[43]. Mean progression-free survival was 4.9 and 46.5 mo for the thrombocytosis and normal platelet groups respectively. In this study prognosis was even better in the sub-group that retained a normal platelet count after the surgery.

In contrast to the above studies, a study that included pancreatic, duodenal and bile duct ampullary carcinomas found lower platelet counts to influence adversely overall and disease-free survival[44]. Lower pre-operative platelets counts were significantly associated with positive surgical margins, a fact that may at least partially explain the adverse prognostic association. Another explanation for this reverse association compared with the previously discussed studies is that this study used a lower cut-off to define high platelet counts at 300 × 10^9/L. The same cut-off of 300 × 10^9/L was used in another more extensive series of 205 patients exclusively with pancreatic adenocarcinoma that had negative results for an association of platelet counts with survival[45]. In both of these studies, results might have been blurred by inclusion of a significant number of patients with higher normal spectrum platelet number in the group of increased platelet counts.

THROMBOCYTOSIS IN HEPATOCELLULAR CARCINOMA

Platelets have a complex relationship with hepatic malignancies. On one hand, due to its association with cirrhosis hepatocellular carcinoma is often presenting with thromcytopenia which is also an adverse prognostic factor[46]. On the other hand, thrombopoietin, an important cytokine for thrombopoiesis, is produced by the liver and may lead to thrombocytosis if neoplastic cells mimic their normal counterparts and produce the cytokine[47] or alternatively if cancer cells stimulate normal liver to produce it[48]. An association of extreme thrombocytosis with both hepatocellular carcinoma and the childhood liver tumor, hepatoblastoma has been noted in the pediatric population[49]. Hepatoblastoma patients had significantly elevated levels of thrombopoietin compared to controls but only slightly elevated levels of IL-6 suggesting that thrombopoietin is down-stream to IL-6 in the pathway triggering thrombopoiesis[50]. Hepatocellular carcinoma patients with thrombocytosis have bigger tumors and a better liver function than patients with normal platelets[51]. A large study of 1154 patients disclosed a 2.7% incidence of thrombocytosis in hepatocellular carcinoma[52]. In addition, platelet count and thrombopoietin level correlated with effectiveness of treatment, decreasing after excision of the tumor and re-increasing upon recurrence. Thrombocytosis was significantly associated with younger age of the patients, higher tumor burden, development of portal

vein thrombosis by tumor involvement and a shorter mean survival time of less than 5 mo as opposed to over 12 mo in patients without thrombocytosis.

THROMBOCYTOSIS IN COLORECTAL CANCER

Thrombocytosis (more than 400×10^9/L) was evaluated as a prognostic factor in an extensive series of 1513 patients with localized colorectal cancer that had undergone surgery[53]. Patients with thrombocytosis had a significant worse overall survival than patients with normal platelets. Overall recurrence rate and distant metastatic recurrence but not loco-regional recurrence was worse in patients with thrombocytosis. These negative effects of thrombocytosis in overall survival and distant metastatic recurrence persisted over a 5 years period from surgery[53].

A retrospective series of 150 patients that underwent surgery for colorectal carcinoma disclosed that patients with pre-operative thrombocytosis had a 5-year survival of 13.3% while patients with normal count pre-operatively had a 5-year survival of 56.3%[54]. Thrombocytosis, together with lymph node positivity, increasing stage and presence of perineural invasion was statistically associated with worse survival. An association of thrombocytosis with survival or cancer specific survival in colorectal cancer was confirmed in two other larger series of 453 and 636 patients from Japan[55,56] and a smaller series of 180 patients from Europe[57]. The authors of one of these studies examined also thrombocytosis specifically in rectal cancer patients receiving chemo-radiotherapy[58]. They reported that patients with thrombocytosis before combined treatment had a lower rate of radiographic and pathologic response to treatment and a higher risk of local recurrence. In another study focusing in rectal cancer, patients with pre-operative thrombocytosis (more than 350×10^9/L) had a significantly worse survival than patients with lower counts[59].

Patients with node negative colorectal cancer represent a particular challenge for the medical oncologist because, although they have a risk for recurrence, they derive no clear benefit from chemotherapy as a whole group. Clinicopathologic characteristics such as T3 invasion, less complete lymph node dissection, high grade and clinical presentation with obstruction or perforation are used to assist in defining the need for adjuvant chemotherapy[60]. In node negative patients additional prognostic markers to guide therapeutic decisions would be particularly valuable. Thrombocytosis could be such a marker and it was found in an investigation of 198 patients with node negative disease to be associated with significantly worse survival than counterparts with normal pre-operative platelet counts[61]. In these node negative patients, thrombocytosis (platelet count more than 400×10^9/L) was independently associated in multivariate analysis, together with tumor depth (T stage), grade and lymphatic invasion, with both disease-free and overall survival.

In contrast to all the above investigations, a single study of 630 patients did not find a correlation of thrombocytosis with survival[62]. This study used a more stringent definition of thrombocytosis of platelet counts of more than 450×10^9/L and included patients of all stages. Inclusion of metastatic patients might have made the effect of platelet counts on outcome more difficult to discern. Despite this, the *P* value in the Cox multivariate model was just outside significance at 0.06[62].

CONCLUSION

Thrombocytosis occurs in a significant minority of patients with cancer and reflects the increase of thrombopoiesis-inducing cytokines in the tumor milieu. Thus it carries an adverse prognostic value both because of this reflection but also because platelets actively promote carcinogenesis and metastasis protecting tumor cells in their metastatic transit and providing bioactive molecules released upon activation in the tumor and metastatic sites. In the gastrointestinal tract, inflammation and infection play a significant role in carcinogenesis with several well-known associations such as inflammatory bowel disease and colorectal cancer, *Helicobacter pylori* infection and gastric cancer and viral hepatitis infection and hepatocellular carcinoma. In addition even in inflammation-independent cancers, cancer-associated molecular lesions may induce platelet-inducing cytokines. For example one of the most common colorectal cancer lesions, Smad4 mutations, lead to dysfunctional TGFβ signaling, resulting in its turn to increased IL-6 signaling[63]. Given these data, a combined treatment blocking IL-6 with the IL-6 monoclonal antibody inhibitor siltuximab or the IL-6R inhibitor tocilizumab together with an anti-platelet function inhibitor such as aspirin with or without inhibition of additional pathways activated by platelet granules cargo factors such as the CXCL12/CXCR4 or the VEGF/VEGFR axis could be a viable option for development in gastrointestinal cancer patients with thrombocytosis to improve their prognosis. Given its significance as a prognostic factor in gastrointestinal cancers and the ease and standardization of its measurement in the clinic, thrombocytosis should be considered as a factor in the stratification process of randomized trials in these cancers, as both a measure of the tumor inflammatory status but also an active propagator of the neoplastic process. Another emerging concept is that of thrombocytosis as a predictor of response to targeted treatments, for example of anti-VEGF therapies. A study in metastatic renal cell carcinoma has shown that patients with thrombocytosis had a higher risk to present a primary refractoriness to anti-VEGF treatments (OR = 1.7, $P = 0.0068$) than patients with normal platelets[64]. It remains to be seen if thrombocytosis could be a predictive factor for anti-VEGF therapies in gastrointestinal cancers and in particular colorectal cancer and hepatocellular carcinoma where the anti-VEGF monoclonal antibody bevacizumab and the small molecule inhibitor sorafenib are clinically used[65,66].

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MINIREVIEWS

Mechanisms linking dietary fiber, gut microbiota and colon cancer prevention

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Abstract

Many epidemiological and experimental studies have suggested that dietary fiber plays an important role in colon cancer prevention. These findings may relate to the ability of fiber to reduce the contact time of carcinogens within the intestinal lumen and to promote healthy gut microbiota, which modifies the host's metabolism in various ways. Elucidation of the mechanisms by which dietary fiber-dependent changes in gut microbiota enhance bile acid deconjugation, produce short chain fatty acids, and modulate inflammatory bioactive substances can lead to a better understanding of the beneficial role of dietary fiber. This article reviews the current knowledge concerning the mechanisms *via* which dietary fiber protects against colon cancer.

Key words: Dietary fiber; Gut microbiota; Colon cancer

Core tip: Dietary fiber modulates our health at nearly every level, and in every organ system, *via* complicated modes of action. This article reviews the mechanistic association of dietary fiber, gut microbiota and colon cancer prevention.

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INTRODUCTION

Colon cancer is one of the most common malignancies in the United States and accounts yearly for approximately 11% of all cancer deaths[1]. The incidence rates of colon cancer are higher in the Western world but are rapidly increasing in developing countries, and it is predicted that half of the Western population will develop at least one colorectal tumor by age of 70[1]. Although cancer treatments have made large strides in recent decades, prevention by diet and other healthy lifestyle factors and habits (*e.g.*, physical exercise) offers a more desirable alternative. Genetic variation and environmental exposures (*e.g.*, diet, physical activity), including diet, are the two main contributing factors influencing the occurrence of colon cancer[2]. Thus, colon cancer may be highly amenable to prevention through a dietary regimen, and dietary carbohydrates may play a critical role[3]. Carbohydrates can be separated into two basic groups based upon their digestibility in the gastrointestinal (GI) tract[4,5]. The first group is simple carbohydrates such as starch and simple sugars, which are easily hydrolyzed by enzymatic reactions and absorbed in the small intestine. The second group is composed of complex carbohydrates such as cellulose, lignin and pectin which are resistant to diges-

tion in the small intestine and undergo bacterial fermentation in the colon. These complex carbohydrates, referred to as dietary fibers, are found in plants[4,5]. Many studies suggest that there is an association between high dietary fiber intake and a low incidence of colon cancer, and that dietary fiber has anticancer properties[6-8]. Furthermore, the US Food and Drug Administration has approved health claims supporting the role of dietary fiber in cancer prevention[9].

It is known that the human GI tract represents the most abundant reservoir of microbes with over 100 trillion bacteria grouped in about 1000 species[10,11]. The bacterial gut populations can be shifted to a healthier composition by fermentable dietary fiber that provides substrates for bacterial fermentation[10,11]. Dietary fiber decreases the risk for type 2 diabetes mellitus, obesity, cardiovascular disease, colon cancer, and improves immunity by modulating the gut microbiota landscape[6]. Dietary fiber modulates our health at nearly every level, and in every organ system, *via* complicated modes of action, many of which remain to be determined[10,11]. In the present review, we focus on the mechanistic association of dietary fiber, gut microbiota and colon cancer prevention.

IMPACT OF DIETARY FIBER ON GUT MICROBIOTA

Dietary fiber constitutes a spectrum of non-digestible food ingredients including non-starch polysaccharides, oligosaccharides, lignin, and analogous polysaccharides with an associated health benefit[12,13]. Dietary fibers are not a static collection of undigestible plant materials that pass through the human GI tract without any function; instead, they bind potential nutrients, result in new metabolites, and modulate nutrient absorption/metabolism. Certain dietary fibers are fermentable, and in addition to their anaerobic degradation in the GI tract, there is also a concurrent anaerobic proteolytic fermentation[14]. Whereas the main fermentation products of fiber are thought to be beneficial (positive), the products of the proteolytic fermentation can be detrimental (negative), resulting in a ying-yang effect[14]. In healthy individuals, fermentation processes are primarily controlled by the amount and type of substrates accessible to bacteria in the colonic ecosystem[11]. The fate of fiber in the colon largely depends on the colonic microbiota and the physio-chemical characteristics of the fiber itself[15]. Fiber sources such as oat bran, pectin, and guar are highly fermented; whereas, cellulose and wheat bran may be poorly fermented[15,16]. On the other hand, the type of dietary fiber affects the microbial composition of the gut lumen. For example, inulin, a polymer of fructose monomers present in onions, garlic and asparagus[17], stimulates the growth of *Bifidobacteria*; whereas, it restricts the growth of potential pathogenic bacteria such as *E. coli, Salmonella,* and *Listeria*[17-19]. In experiments with a simulator of the human colon, dietary xylo-oligosaccharides decrease the major butyrate-producing bacteria *Faecalibacterium prausnitzii*, although total butyrate concentration is increased only in the distal vessel[20]. The same researchers reported that xylo-oligosaccharides also affect the levels of sulphate-reducing bacteria, *Bacteroides fragilis*, providing evidence that dietary carbohydrates modify the gut microbiota, and therefore, its ability to change the physiological properties of the colonic environment. In humans, diets high in nonstarch polysaccharides and/or resistant starch profoundly affect the types of fecal bacteria, including species related to *Ruminococcus bromii*, which can contribute to starch degradation and short chain fatty acid (SCFA) production[21].

There are over 50 bacterial phyla described to date but the human gut microbiota is dominated by two of them, the *Bacteroidetes* and the *Firmicutes*; whereas, the phyla *Proteobacteria, Verrucomicrobia, Actinobacteria, Fusobacteria, and Cyanobacteria* are present in minor proportions[22,23]. The taxonomic composition of the "ideal" microbiota, if such exists, remains to be identified. Presently, individuals are categorized into "enterotypes" or clusters based upon the abundance of key genera in the gut microbiota[24]. Recent studies showed that gut microbial communities are clustered into three types: *Bacteroides* (enterotype 1), *Prevotella* (enterotype 2) and *Ruminococcus* (enterotype 3), and these clusters seem unrelated to geographical origin, body mass index, age, or gender[25]. These findings suggest that there is not one ideal microbiota composition, but "a limited number of well balanced host-microbial symbiotic states"[25].

Much remains to be determined about what constitutes a healthy microbiota, but there are numerous diseases and conditions associated with a disturbed gut microbiota[26]. It has been generally accepted that the human gut contains approximately 500 to 1000 species[27], and the differential colonization suggests a relationship with disease susceptibility[28-30]. For example, the intestinal microbiota of children from Europe and rural Africa who are exposed to a modern Western diet and a rural diet respectively, exhibit significant differences in microbial composition. The major difference is that rural African children have microbiota enriched in *Bacteroidetes* and depleted in *Firmicutes* in comparison to European children[30].

Although amino acid fermenting bacteria and syntrophic species are present in the large intestine, the majority of colonic bacteria have predominantly saccharolytic metabolisms. Therefore, dietary fiber/carbohydrate availability is almost certainly the most important nutritional factor that determines the composition and metabolic activities of the gut microbiota, and many of the physiologic properties of the microbiota are attributed to the fermentation and production of SCFAs[31]. For example, lower dietary fiber intake and consistently lower SCFA production were observed in colon cancer risk subjects compared to healthy individuals, and these differences were accompanied by distinct profiles of

the fecal microbiota communities of the two groups[32]. In the same study, *Clostridium*, *Roseburia*, and *Eubacterium spp*. were significantly less prevalent in the colon cancer risk group than the healthy individuals group; whereas, *Enterococcus* and *Streptococcus spp*. were more prevalent in the colon cancer risk group[32]. Consistent with these observations, the low pH conditions resulting from fiber fermentation increase biosynthetic requirements for nitrogen-containing precursors, and subsequently inhibit toxin accretion in the colon[33]. Taken together, individual properties such as body mass index, age, or gender may not explain the three observed gut bacterial enterotypes[25], but data-driven marker genes/microbial markers can be identified for certain diseases and conditions[30-32].

SCFA PRODUCTION

Dietary fiber consumption can have significant health benefits, particularly in laxation, mineral absorption, potential anticancer properties, lipid metabolism and anti-inflammatory effects[34]. Many of these health benefits can be attributed to the fermentation of dietary fiber into SCFAs in the colon. These SCFAs are generated by the colonic microbiota, and an equation outlining overall carbohydrate fermentation in the colon has been described[35]:

$59\ C_6H_{12}O_6 + 38\ H_2O \rightarrow 60$ acetate $+ 22$ propionate $+ 18$ butyrate $+ 96\ CO_2 + 256\ H^+$.

The significance of carbohydrate breakdown by intestinal bacteria is broad. For example, the increased input of carbohydrates allows for increased bacterial cell mass, which supports laxative effects and shorter colonic transit times. The decreased transit times decrease protein breakdown and the accumulation of putrefactive substances, such as ammonia, phenols, amines and hydrogen sulfide in the colon.

The three major colonic SCFAs are acetate, propionate and butyrate, and the total concentration of SCFAs in colonic content may exceed 100 mmol/L [36]. The composition of diet and gut microbiota are the major factors in determining the molar proportion of SCFA species. In general, acetate makes up around 60%-75% of the total SFCA, and is generated by many of bacterial groups that inhabit the colon, with approximately one-third of the product coming from reductive acetogenesis[37]. The bacterial groups that form propionate and butyrate are specialized, and are of particular interest in terms of their health beneficial effects. The fact that a considerable number of bacterial species provide diverse molecular functions underscores the importance of a functional analysis to understand the composition of microbiota[25].

The data on the main propionate-producing bacteria in the human colon are still emerging, and several biochemical pathways for propionate formation are characterized[38,39]. The succinate route for propionate formation is generally employed by *Bacteroides* species, but the acrylate route from lactate is adopted by bacteria belonging to the clostridial cluster IX group. In addition, a third pathway is employed by the butyrate-producing bacterium *R. inulinivorans* with fucose as substrate[40].

Colonic bacteria that produce butyrate belong to the clostridial clusters I , III, IV, VI, XIVa, XV and XVI. Two particularly abundant groups that are estimated to consist 7%-24% of the total gut bacteria in healthy subjects are cluster IV bacteria related to *Faecalibacterium prausnitzii*, and cluster XIVa bacteria related to *Eubacterium rectal* and to *Roseburia spp*[41]. For example, reduced dietary intake of fiber by obese subject results in decreased concentrations of butyrate and butyrate-producing bacteria related to *Eubacterium rectal* and to *Roseburia spp*[42].

PHYSIOLOGICAL EFFECTS OF SCFA

Acetate (C2), propionate (C3) and butyrate (C4) are found in the human intestine at concentrations of approximately 13 mmol/L in the terminal ileum, approximately 130 mmol/L in caecum and approximately 80 mmol/L in the descending colon[36]. These SCFAs released in the intestinal lumen are readily absorbed and used as energy source by colonocytes (approximately 10% of basal energy requirements) and also by other tissues such as liver and muscle[43].

Acetate stimulates proliferation of normal crypt cell but reduces the frequency of spontaneous longitudinal muscle contractions in rat colonic smooth muscle[44]. Acetate enhances ileal motility, increases colonic blood flow, and plays a role in adipogenesis and host immune system through interacting with the G protein-coupled receptor (GPCR43, 41) in adipose tissue and immune cells[45,46]. In addition, it has been shown that acetate reduces lipopolysaccharide-stimulated tumor necrosis factor (TNF), interleukin (IL)-6 and nuclear factor (NF)-κB level while boosting peripheral blood antibody production in various different tissues[47].

Similar to acetate, propionate has been shown to exert a concentration-dependent effect on the frequency of spontaneous contractions in longitudinal muscle *via* enteric nerves in rat distal colon[44]. In both animal and human studies, it has been shown that propionate reduces food intake and increases satiety *via* augmentation of the satiety hormone leptin, and through activation of GPCR43, 41[48,49]. Also, propionate may be protective against carcinogenesis because it reduces human colon cancer cell growth and differentiation *via* hyperacetylation of histone proteins and stimulation of apoptosis[50,51]. In addition, propionate also inhibits the production of pro-inflammatory cytokines (*e.g.*, TNF-α, NF-κB) in multiple tissues[52,53].

Although acetate, propionate, and butyrate are all metabolized to some extent by the epithelium to provide energy, butyrate plays the most critical role in maintaining colonic health and moderating cell growth and differentiation[54]. More than 70% of oxygen consumption in isolated colonocytes is due to butyrate oxidation, and the uptake and utilization of butyrate by the colonic epithelium have been demonstrated in a study on the SCFA lev-

els in portal and arterial blood and in colonic contents[36]. Compared to acetate and propionate, butyrate exhibits strong anti-inflammatory properties, and this effect is likely mediated by inhibition of TNF-α production, NF-κB activation, and IL-8, -10, -12 expression in immune and colonic epithelial cells[55,56].

ANTI-INFLAMMATORY ACTION, SCFAS AND MICROBIOTA

Inflammation, a host defense mechanism, is an immediate response of the body to tissue injury caused by microbial infection and other noxious stimuli. However, inadequate resolution of inflammation and uncontrolled inflammatory reactions can evoke a state of chronic inflammation, which is a common etiologic factor for cancer[57].

Leukocyte recruitment and SCFAs

Leukocytes are recruited and migrate from the bloodstream to the inflamed tissue through a multistep process that involves expression and activation of several proteins such as adhesion molecules and chemokines[58], and SCFAs modify this leukocyte recruitment[59,60]. Several lines of evidence show that SCFAs induce directional migration of neutrophils, which is dependent upon the activation of GPR43, a G protein-coupled receptor[59,61]. The function of SCFAs as agonists of GPR43 may result in activation of protein kinase B (PKB) and mitogen activated protein kinases in neutrophils. Furthermore, the receptors GPR41 and GPR109A, both of which are related to GPR43, are activated by SCFAs[62]. These results support a role for the SCFAs in the movement of neutrophils[61].

SCFAs also modulate the expression and secretion of cell adhesion molecules and chemokines that play a central role in leukocyte recruitment[52,60]. Cell adhesion molecules such as selectins, integrins, vascular cell adhesion molecule-1, and intercellular adhesion molecule-1 are critical for adhesion and transendothelial migration of leukocytes[63]. Recent studies have shown that SCFAs reduce the adherence of monocytes and lymphocytes to human umbilical vein endothelial cells, and this is associated with an attenuation of NF-κB and PPARγ activities and adhesion molecule expression (ICAM-1 and VCAM-1)[52,63]. In addition, butyrate reduces the constitutive and IFN-γ-induced expression of LFA-3 and ICAM-1; the LPS-stimulated production of CXCL-2, 3, and macrophage chemoattractant protein-1, IL-8 by neutrophils and macrophages[64,65]. Therefore, by modulating the amount or type of adhesion molecules and chemokines, SCFAs may alter the recruitment of leukocytes, and in part, reduce the chronic GI tract inflammatory response.

Proinflammatory mediators, SCFAs

A wide variety of cytokines and other proinflammatory mediators contribute to both extrinsic and intrinsic pathways of inflammation-associated carcinogenesis, and macrophages are the major source of inflammatory mediators[57]. Once activated, macrophages produce significant amounts of mediators such as TNF-α, Il-1β, IFN-γ and IL-6, chemokines, and nitric oxide (NO)[57,66]. SCFAs, mainly butyrate, reduce the LPS- and cytokine-stimulated production of pro-inflammatory mediators such as TNF-α, IL-6, IFN-γ and NO while increase the release of the anti-inflammatory cytokine IL-10[66,67]. The histone deacetylases (HDACs) and histone acetyltransferases control the degree of protein acetylation and gene expression, and the ability of butyrate to inhibit HDAC activity is the main mechanism *via* which the acid affects the expression of proinflammatory mediators[66-68]. In addition to increasing net histone acetylation and therefore, influencing gene expression, butyrate also augments the acetylation of nonhistone proteins such as NF-κB, MyoD, and p53[66].

Gastrointestinal barriers and microbiota

Gut microbiota contribute to the maintenance of an intact GI barrier, and the disruption of this barrier can cause an inflammatory process[10]. The primary or innate barrier is an interaction between the microbiota and the gut epithelial cell layer. This interaction is an active process, in which certain inflammatory mediators are produced. For example, the ligands of toll like receptors (TLRs) such as LPS and flagellin are microbially derived, and they activate respectively, TLR-4 and -5 to modulate distinct aspects of host metabolism and immune response[69]. The secondary physical barrier is formed by epithelial cell secretion of mucus, and this intestinal mucus layer is a critical physical barrier protecting the intestinal epithelium from the intestinal microbiota, including invasive microbes[70]. The mucus layer is composed by mucin proteins produced by Goblet cells[10], whereas, in the small intestine, the Paneth cells directly sense enteric bacteria through TLR activation, and release various antimicrobial peptides[71]. Therefore, mucus not only forms a physical barrier and provides a nutrition source for the microbiota, but it also contains protective mediators such as secreted antimicrobial peptides and Ig A[70,72]. Thus, the mucosal immune system and the homeostasis of gut microbiota are interdependent, and a balance between them maintains a stable intestinal environment.

EFFECT OF SCFAS ON CELL CYCLE, MIGRATION AND APOPTOSIS

Although SCFAs stimulate normal colonocyte proliferation at low concentrations (*e.g.*, 0.05 mmol/L-0.1 mmol/L butyrate), SCFAs also inhibit the growth of most human colon cancer cells by cell cycle arrest and apoptosis through a complex molecular regulation[73,74]. Several *in vitro* studies have demonstrated that butyrate inhibits HDACs, and allow histone hyperacetylation that leads to transcription of many genes including p21/Cip1, and cyclin D3[75]. The induction of the cyclin-dependent kinase

inhibitory protein p21/Cip1 accounts for cell arrest in the G_1 phase of the cell cycle[75]. In addition, we and others have also observed that at 0.5 or higher mmol/L concentration, butyrate inhibits the migration and invasion rate of cancer cells by increasing the expression of anti-metastasis genes (*e.g.*, metalloproteinases) and inhibiting the activation of pro-metastatic genes (*e.g.*, matrix metalloproteinases)[76,77].

There is also overwhelming evidence that dietary fiber counteracts the earliest stages of colonic carcinogenesis. For example, carbohydrates may protect colonocytes against the genotoxicity of a typical Western diet, which is characterized by increased levels of protein and fat intake. Thus, resistant starch decreases by 70% the DNA damage manifested by single-strand breaks in colonocytes of rats fed a Western diet[78]; significantly, when such DNA damage is not repaired, it may initiate colonic carcinogenesis. This interpretation is supported by experimental data that resistant starch protects rodents against tumors induced by the carcinogen azoxymethane[79,80]. The protective effect of resistant starch against such DNA alterations could be attributed to the increased production of SCFAs, and the decreased phenol and ammonia levels[78]. Among the SCFAs, butyrate has been demonstrated to have a significant physiological effect on neoplastic colonic cells[81]; however, acetate has also been implicated in protection against genotoxic agents[20] Interestingly, different carbohydrates affect differentially the extent of DNA damage; for example, dietary xylo-oligosaccharides but not inulin may alter the genotoxicity of the colonic environment. Utilizing a human colonic simulator inoculated with human feces and a soy protein isolate, the researchers have reported that xylo-oligosaccharides reduce genotoxicity of the liquid phase in the proximal vessel, but increase genotoxicity in the distal vessel[20].

It is evident that the DNA-protective effects of the carbohydrates are mediated by (1) their ability to sustain the existence of specific colonic microbiota; and (2) by the fermentation products resulting from the presence of the colonic bacterial species. In rats, a resistant starch-enriched diet increases the numbers of bifidobacteria and lactobacilli species; whereas, it decreases coliforms and results in higher levels of SCFAs[82]. However, the levels of the short-chain fatty acids are dependent not only upon the type and amount of dietary carbohydrates, but also by the present colonic bacterial species. Such two-way interactions explain the observations that rats fed resistant starch diet supplemented with the probiotic *Bifidobacterium lactis* exhibit a stronger apoptotic response to a genotoxic carcinogen in the colon than those fed the same diet without the probiotic supplement[82].

Evidence for a protective role of butyrate against colon cancer comes mostly from studies in carcinogen-induced rodent models of this malignancy. Thus, the effects of diets containing guar gum and oat bran (both highly fermentable, but associated with low butyrate levels in the distal colon) have been compared to these of a diet with wheat bran (resulting in high butyrate concentrations) in a rat dimethylhydrazine model of colon cancer[83]. The researchers reported the highest protection against colonic tumors in the group of rats fed the wheat bran diet. Similarly, rats fed diet with resistant starch exhibited a lesser burden of colonic adenocarcinomas after exposure to azoxymethane, and this protective effect seemed to be related to the production of butyrate in the colon[79]. It has been observed that in rats with tumors induced by azoxymethane and deoxycholic acid, dietary sodium gluconate increases butyrate levels and decreases the numbers of tumors in the colon[84]. Also, oral administration of the butyrate-producing bacteria *Butyrivibrio fibrisolvens* augmented butyrate levels, and reduced the formation of aberrant crypt foci, an early colonic lesion, in the colon and rectum of mice treated with dimethylhydrazine[85].

However, not all reports support a chemopreventive effect for butyrate[15]. Some epidemiological studies have also shown no relationship between fiber intake and colon cancer incidence, and no effect of SCFAs (*e.g.*, butyrate) on colonic tumorigenesis[86,87]. These observations were initially counter-intuitive given the reported anticancer-effects of dietary fiber/SCFAs. However, molecular analyses on the effect of SCFAs in colonic tumorigenesis may partly explain these seemingly controversial observations.

First, the constitutive activation of the canonical WNT signaling pathway is a common characteristic of colon cancer, and the beta-catenin- Tcf (BCT) transcriptional complexes are the downstream mediators of this pathway[88,89]. It has been proposed that WNT/beta-catenin activity exists as a gradient, within which absence of WNT signal results in terminal differentiation and apoptosis, relatively low levels of signaling lead to controlled self-renewal, moderate levels of signaling promote uncontrolled cell proliferation, and relatively high levels of WNT signaling lead to apoptosis[90]. Therefore, hyper-activation of WNT/beta-catenin signaling in butyrate-treated colon cancer cells is a required event to achieve high levels of apoptosis in these cells[91].

Second, studies on human colon cancer cell lines with different WNT/beta-catenin signaling mutations have identified two classes of cell lines: those which respond to butyrate treatment with (1) a high fold; and (2) a low fold induction of WNT/beta-catenin activity and apoptosis[91]. Thus, discrepancies in the literature as to the protective nature of fiber intake against colon cancer[5,15,92] may be due to the fact that only a subset of colonic lesions responds to butyrate with hyper-activation of WNT/beta-catenin signaling and enhanced apoptosis. Further, colonic lesions may become resistant to the effects of butyrate through exposure to suboptimal levels of this agent; for example, butyrate-resistant cells produced *in vitro* exhibit suppressed WNT/catenin hyperactivation and inhibited induction of apoptosis upon exposure to butyrate and other HDAC inhibitors[93]. This butyrate-resistant cell line may reflect the *in vivo* existence of human tumors that are

resistant or partially resistant to the effects of butyrate, and suggests that a high dietary fiber intake is required for an effective protective action against colon cancer. Differences in the responsiveness of colonic neoplastic cells to the effects of butyrate on WNT/catenin signaling may be mediated through the differential expression and activity of transcriptional coactivators that influence WNT/catenin activity, particularly CBP and p300[94,95]. For example, a butyrate-resistant cell line has been shown to be defective in p300 expression, which likely mediates effects of butyrate on WNT/catenin signaling and cell physiology[95].

Third, the composition of gut microbiota and diet (*e.g.*, fat) are factors that affect the SCFA productions and their action[15,96,97], and the effect of SCFAs on colon neoplastic cells might be modifiable by other dietary compounds and metabolites; thus, adding a particular type of oil (*e.g.*, fish oil *vs* corn oil) results in a variable reduction of colon tumors in rat azoxymethane model of carcinogenesis[98]. Finally, the effect of fiber and butyrate on colon carcinogenesis is likely dependent upon the timing of fiber and butyrate administration with respect to the stage of cancer development[15]. Several studies have shown that a high fiber intake specifically affects early tumor development in the colon; however, progression to advanced adenomas is unlikely to be influenced by fiber intake[7,86]. These data clearly support a multifaceted role of SCFA production/action, and more *in vivo* studies are warranted to further dissect the role of fiber intake in modulating colon cell cycle and apoptosis pathways.

FUNCTIONAL ROLE OF FIBER SOURCE PER SE

Although gut microbiota and fiber fermentation to SCFAs play a critical role in cancer prevention, the fiber source per se may have independent effects on colonic health. First, dietary fiber increases viscosity and fecal bulking (diluting potential carcinogens), and it therefore shortens the time for proteolytic fermentation (and production of harmful substances) and also decreases the contact between potential carcinogens and mucosal cells[4,99]. In addition, dietary fiber could bind/excrete potential luminal carcinogens (*e.g.*, secondary bile acids) and lower fecal pH in the colon[4,100,101]. Second, dietary fiber is not only a substrate for fermentation, but it is also a source of vitamins, minerals and slowly digestible energy; for example, bran fractions are rich in minerals, vitamin B6, thiamine, folate and vitamin E[102]. Third, dietary fiber is associated with phytochemicals such as phenolics, carotenoids, lignans, beta-glucan and inulin[102,103]. For example, arabinoxylan, a constituent of hemicelluloses, is an important source of phenolic compounds that may be released in the colon during fermentation of complexed fibers[4,102]. These bioactive substances may protect the GI tract from oxidative damage, although this possibility is controversial due to the anaerobic environment in the colon and the fact that the fiber-associated phytochemicals (*e.g.*, carotenoids) do not seem to be absorbed through the GI tract into the rest of the body, even though the colon is the primary site for fiber fermentation and the release of these chemicals[104]. However, since the concentrations of bioactive substances derived from dietary fiber sources can be much higher in the colonic lumen than in plasma and other tissue, these phytochemicals may delay the onset of colon cancer.

CONCLUSIONS AND PERSPECTIVES

A large amount of research has reported an inverse relationship between dietary fiber intake and colon cancer risk. The protective effect of fiber against colon cancer derives from a multi-layered system of mechanistic checks and balances, which may explain why not all studies report this beneficial effect. Although the anticancer mechanisms of dietary fiber are not fully understood, several modes of action have been proposed (Figure 1). First, dietary fiber resists digestion in the small intestine, and enters the colon where it is fermented to produce SCFAs that may enhance the healthy composition of gut microbiota. Second, SCFAs have anticancer properties which include the promotion of cancer cell cycle arrest, apoptosis, and the inhibition of chronic inflammatory process and cancer cell migration/invasion in the colon. Importantly, these molecular activities are effective only within a certain physiological concentration range of the SCFAs. Third, dietary fiber increases fecal bulking and viscosity, reduces the time for proteolytic fermentation that results in harmful substances, and shortens the contact between potential carcinogens and mucosal cells. In addition, dietary fiber can bind/excrete potential luminal carcinogens (*e.g.*, secondary bile acids), lower fecal pH in the colon, and thus provide a healthy intestinal environment.

Not all fibers have the same properties; therefore, the characteristics and components of dietary fibers (*e.g.*, arabinoxylan, β-glucan) may determine their modes of action against colon cancer cells. Future studies on the type of fiber and fiber components may provide a better understanding of how and why dietary fiber decreases the risk of colon cancer. Furthermore, evidence from many lines of research demonstrates that fiber consumption modifies the composition of gut microbiota, and a well balanced colonic microbiota influences the host at nearly every level including immunity and neoplastic development. Metagenomics is one of the newest approaches to determine gut microbiota composition, but it is still difficult to characterize the interactions between hosts and their microbiota. The combination of several "meta" analyses such as metagenomics, metabolomics, metatranscriptomics, and the shift of focus from a "who is there" to a "why are they there" will advance our understanding of the relationship between dietary fiber consumption, microbiota composition, and human health. Future studies are required to unravel the microbiota changes that correlate with the beneficial effects of fiber, although it is likely that such changes in the gut bacteria may be dose-,

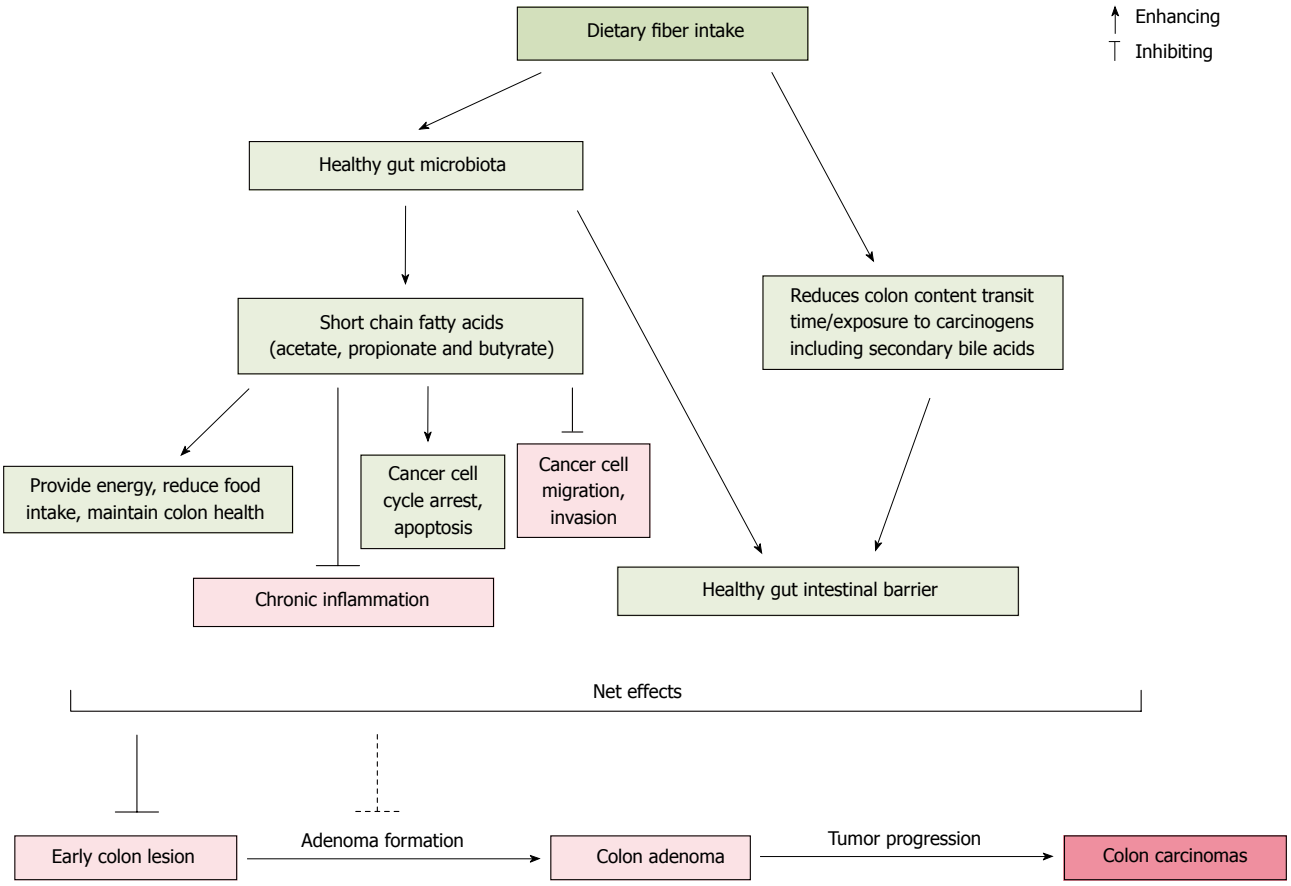

Figure 1 The proposed interaction of primary pathways related to dietary fiber consumption, gut microbiota and colon cancer risk.

time-, and strain-dependent. These efforts may lead to identification of microbiota signatures that are causal or correlative biomarkers for fiber consumption and colon cancer prevention.

If butyrate is indeed the key mediator for the protective effect of fiber against colon cancer, then the effects of diet and microbiota on the butyrate levels in the colon, and our ability to manipulate these levels *via* dietary supplements, will be important for designing effective colon cancer preventive strategies. The levels of fecal butyrate among individuals differ widely (3.5-32.6 mmol/kg), and these inter-individual differences have been explained in part by body-mass index and dietary intake of protein, fiber, and fat[105]; however, there are additional factors that remain to be determined.

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CASE REPORT

Extended cancer-free survival after palliative chemoradiation for metastatic esophageal cancer

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Abstract

We report on a patient who remained cancer-free for an extended time after palliative radiotherapy (RT) and chemotherapy (nedaplatin plus 5-fluorouracil) treatment for stage Ⅳ (cT3N3M1) esophageal squamous cell carcinoma. Although multiple lymph nodes outside the RT field recurred, the local primary tumor within the RT field did not recur, even 17 mo after palliative RT of 30 Gy in 10 fractions. In this case, acute toxicity, such as myelosuppression or esophagitis, was not enhanced by increasing the fraction dose from 1.8-2.0 Gy to 3.0 Gy. Because 30 Gy in 10 fractions can be completed within a shorter time and is less expensive than 50.4 Gy in 28 fractions, we think that 30 Gy without oblique beams is a more favorable RT method for patients.

Key words: Radiotherapy; Chemotherapy; Esophageal cancer; Esophageal stenosis; Metastatic esophageal cancer

Core tip: The palliative therapy method has not been confirmed for metastatic esophageal cancer. This case report represents a patient who was cancer-free for an extended period of time after palliative chemoradiation of 30 Gy in 10 fractions. We think that 30 Gy without oblique beams is a more favorable radiotherapy method for patients.

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INTRODUCTION

Esophageal cancer, which has the highest incidence and mortality worldwide, is one of the most common malignant tumors in Japan. Japan is recognized as having one of the highest incidence rates of esophageal squamous cell carcinoma in the world.

Due to a lack of obvious early symptoms, patients are often diagnosed at advanced stages, and more than half of patients present with metastases[1]. The recurrence and metastasis rates of esophageal cancer after treatment have tended to increase in recent years. In 2007, Grünberger *et al*[2] confirmed that palliative chemotherapy can prolong the survival of stage Ⅳ esophageal cancer patients, relieve their symptoms and improve their quality of life. Esophageal squamous cell carcinoma is the most common histology in Japan, and its constituent ratio is different from that in Europe and America.

Most patients with esophageal cancer present with dysphagia, and more than half of the patients have inoperable disease at the time of presentation[3,4]. The pri-

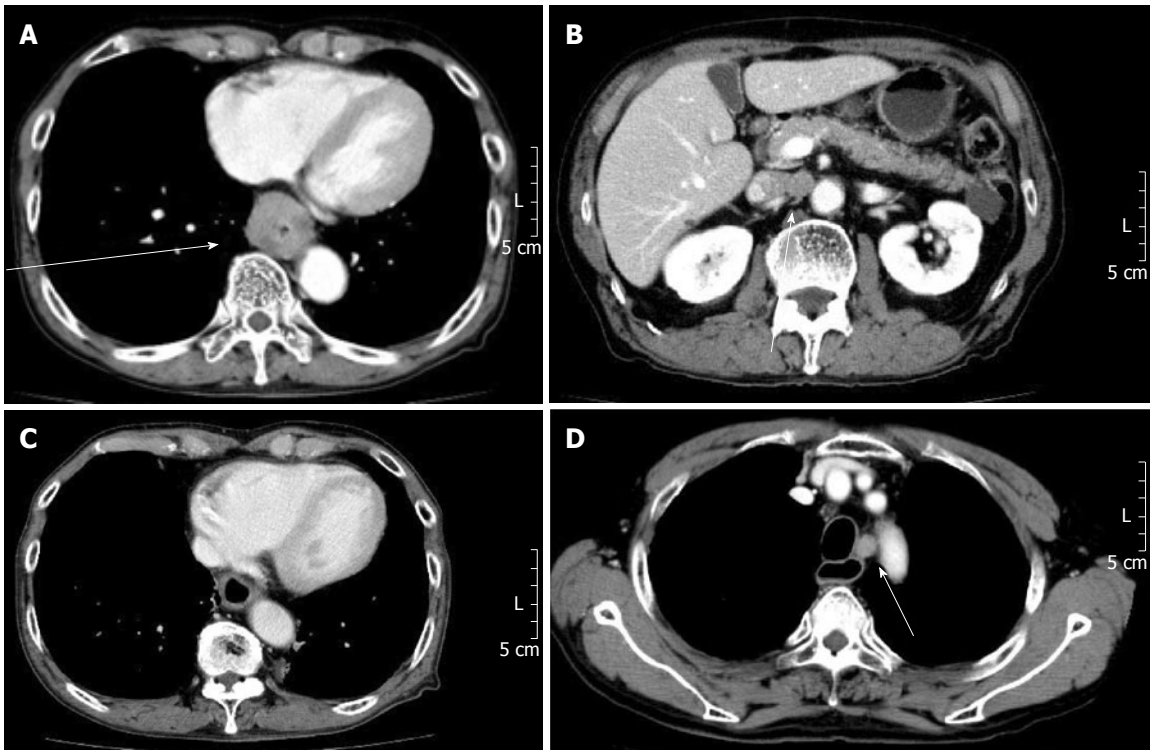

Figure 1 Computed tomography. A: Circumferential wall thickening and a 39 mm × 35 mm tumor on the lower thoracic esophagus; B: Swelling of the abdominal para-aortic lymph node; C: A remarkable shrinking of the mass in the lower esophagus; D: Swelling of the left para-tracheal lymph node.

mary aim of treatment in these patients is to relieve the dysphagia with minimal morbidity and mortality and thus improve their quality of life.

We present a case of extended cancer-free survival after palliative radiotherapy (RT) and chemotherapy in a stage IV esophageal squamous cell carcinoma patient.

CASE REPORT

A 76-year-old Japanese man was referred to our hospital after a few months of dysphagia due to esophageal stenosis. A chest X-ray did not show any characteristic malignancy. A gastrofiberscopy and computed tomography (CT) scan showed a circumferential wall thickening and a 39 mm × 35 mm tumor on the lower thoracic esophagus (Figure 1A). A biopsy on December 13, 2011, revealed squamous cell carcinoma. Laboratory findings, including staining for tumor markers, such as p53, and squamous cell carcinoma, were all within the normal ranges, except for the cytokeratin 19 fragments (CYFRA), which were elevated at 4.3 ng/mL (normal 0-2.0 ng/mL, IRMA method). A chest/abdominal CT scan with enhancement on December 5, 2011, revealed multiple lymph node metastases, including the left supraclavicular, tracheal bifurcation, gastric cardia, and abdominal para-aortic lymph nodes (Figure 1B) (cT3N3 M1, c-Stage IV).

It was decided that our patient should undergo chemoradiation therapy (CRT). The patient received 30 Gy in 10 fractions of 3 Gy on the original tumor location using a 2-field technique of external beam irradiation from December 21, 2011, to January 10, 2012. The patient also received nedaplatin chemotherapy at a dose of 80 mg/m^2 (day 1) plus 5-fluorouracil at 800 mg/m^2 per day (days 1-4) starting on December 26, 2011.

A plain chest/abdominal CT scan on January 23, 2012, after a single cycle of chemotherapy, revealed a remarkable shrinkage of the mass in the lower esophagus and in all lymph nodes (Figure 1C). An enhanced chest/abdominal/pelvic CT scan on April 17, 2012 (after 4 cycles), August 20, 2012 (after 7 cycles), and October 16, 2012, revealed that the tumors continued to shrink. After 6 cycles of chemotherapy, the CYFRA levels had decreased to normal by June 15, 2012. One additional cycle of chemotherapy was added on July 17, 2012. After CRT, the patient had regular follow-up appointments every 3-4 mo.

After 8 completely asymptomatic months following chemotherapy and 14 mo after palliative RT, the tumor was found to have recurred during a regular follow-up appointment. A chest and abdominal enhanced CT scan on March 12, 2013, revealed that the metastatic tumor had spread to multiple lymph nodes, including the retro-esophageal, left para-tracheal (Figure 1D), supraclavicular, and bilateral hilum lymph nodes, but local recurrence was not observed. According to a cervical/chest/abdominal enhanced CT scan that was performed on June 11, 2013 (17 mo after palliative RT), local disease remained controlled.

DISCUSSION

More than 50% of patients with esophageal cancer are not amenable to surgical excision at the time of diagno-

sis, because of either advanced disease or the presence of comorbid conditions. For such patients, palliation of the symptoms is the mainstay of treatment[4].

According to the Radiation Therapy Oncology Group 94-05 trial[5], the standard radiation dose for patients with clinical stage T1 to T4, N0/1, M0 esophageal carcinoma that are selected for a nonsurgical approach and concurrent treatment with 5-FU and cisplatin chemotherapy is 50.4 Gy. Additionally, at our institution, 50.4 Gy in 28 fractions is selected as a curative method. In this case, 30 Gy in 10 fractions was selected as a palliative irradiation dose. Although multiple lymph nodes outside the RT field recurred, the local primary tumor within the RT field did not recur, even 17 mo after RT. In this case, acute toxicity, such as myelosuppression or esophagitis, was not enhanced by increasing the fraction dose from 1.8-2.0 Gy to 3.0 Gy. Because 30 Gy in 10 fractions was completed within a shorter time and was less expensive than 50.4 Gy in 28 fractions, we think that 30 Gy without oblique beam is a more favorable RT method for patients. Because control of the primary lesion of esophageal cancer is directly connected to the inability of the patient to ingest and the subsequent QOL deterioration, a total radiation dose of as much as 30 Gy, not 25 or 20 Gy, was used with palliative intent in our institution.

According to Matsumoto *et al*[5], docetaxel and nedaplatin combination chemotherapy with and without radiation therapy is well tolerated (2-year overall survival was 11.1%) and useful as a second-line chemotherapy for patients with relapsed or metastatic esophageal cancer.

COMMENTS

Case characteristics

A 76-year-old male was referred to our hospital with a few month history of dysphagia due to esophageal stenosis.

Clinical diagnosis

A gastrofiberscopy and computed tomography scan showed a circumferential wall thickening and a 39 mm × 35 mm tumor on the lower thoracic esophagus.

Differential diagnosis

Esophageal leiomyoma, polyp, hemangioma, papilloma, lipoma, cyst.

Laboratory diagnosis

Laboratory findings, including tumor markers like p53 and squamous cell carcinoma, were all within normal values, except for cytokeratin 19 fragments, which was raised at 4.3 ng/mL (normal 0-2.0 ng/mL, IRMA method).

Imaging diagnosis

A chest/abdominal computed tomography scan revealed multiple lymph node metastases such as left supraclavicular, tracheal bifurcation, gastric cardia, and abdominal para-aortic lymph node (cT3N3M1, c-Stage Ⅳ).

Pathological diagnosis

A biopsy revealed a squamous cell carcinoma.

Treatment

The patient was treated with 30 Gy in 10 fractions of external beam irradiation and chemotherapy of nedaplatin plus 5-fluorouracil.

Related reports

According to Matsumoto H, docetaxel and nedaplatin combination chemotherapy with and without radiation therapy is well tolerated and useful (2-year overall survival was 11.1%) as second-line chemotherapy for patients with relapsed or metastatic esophageal cancer.

Experiences and lessons

The authors think that 30 Gy without oblique beams is a more favorable palliative radiotherapy method for patients with metastatic esophageal cancer.

Peer review

This article applies a rare case who survived long time after palliative chemoradiation.

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REVIEW

The role of antioxidants and pro-oxidants in colon cancer

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Abstract

This review focuses on the roles antioxidants and pro-oxidants in colorectal cancer (CRC). Considerable evidence suggests that environmental factors play key roles in the incidence of sporadic CRC. If pro-oxidant factors play an etiological role in CRC it is reasonable to expect causal interconnections between the well-characterized risk factors for CRC, oxidative stress and genotoxicity. Cigarette smoking, a high dietary consumption of n-6 polyunsaturated fatty acids and alcohol intake are all associated with increased CRC risk. These risk factors are all pro-oxidant stressors and their connections to oxidative stress, the intestinal microbiome, intestinal microfold cells, cyclooxygenase-2 and CRC are detailed in this review. While a strong case can be made for pro-oxidant stressors in causing CRC, the role of food antioxidants in preventing CRC is less certain. It is clear that not every micronutrient with antioxidant activity can prevent CRC. It is plausible, however, that the optimal food antioxidants for preventing CRC have not yet been critically evaluated. Increasing evidence suggests that RRR-gamma-tocopherol (the primary dietary form of vitamin E) or other "non-alpha-tocopherol" forms of vitamin E (*e.g.*, tocotrienols) might be effective. Aspirin is an antioxidant and its consumption is linked to a decreased risk of CRC.

Key words: Colorectal cancer; Vitamin E; Tocopherols; Tocotrienols; Oxidative stress; Microbiome; Intestinal; Cyclooxygenase-2; Intestinal microfold cells; Alcohol; Cigarette smoke; Antioxidants; Genotoxicity

Core tip: This review summarizes the roles that antioxidant and pro-oxidant factors play in the development of colorectal cancer (CRC). This review is timely since our understanding of the roles these factors play in CRC has made major advances and is now ripe for translational research efforts. A systems biology research approach appears to be optimal since environmental pro-oxidative stress factors (such as cigarette smoking, a high dietary consumption of n-6 polyunsaturated fatty acids and alcohol intake) are likely to interact with the intestinal microbiome causing genotoxic damage to the epithelial cells of the large intestine and CRC.

Stone WL, Krishnan K, Campbell SE, Palau VE. The role of antioxidants and pro-oxidants in colon cancer. *World J Gastrointest Oncol* 2014; 6(3): 55-66 Available from: URL: http://www.wjgnet.com/1948-5204/full/v6/i3/55.htm DOI: http://dx.doi.org/10.4251/wjgo.v6.i3.55

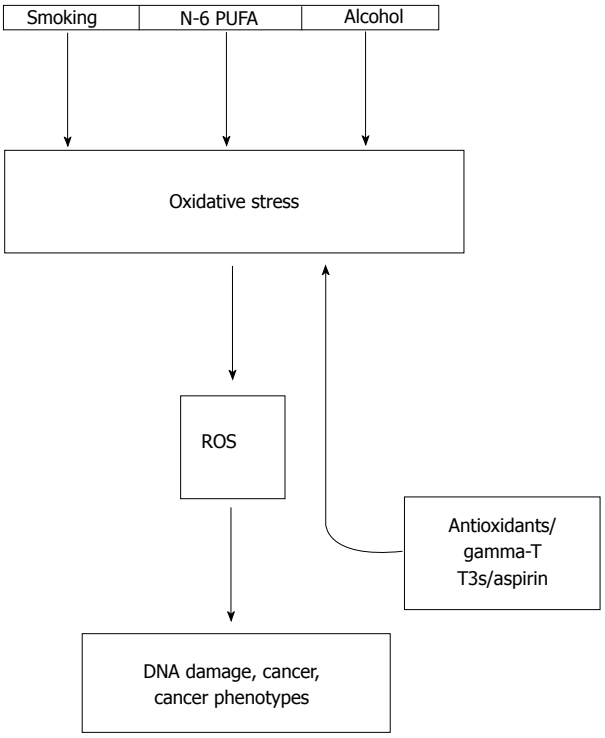

Figure 1 Connections between known risk factors of colorectal cancer and oxidative stress. Smoking, dietary n-6 polyunsaturated fatty acids (n-6 PUFA) and heavy alcohol consumption contribute to in vivo oxidative stress with an accompanying overproduction of genotoxic reactive oxygen species (ROS) that give rise to mutations, cancer and also promote cancer phenotypes. Antioxidants such as gamma-tocopherol (gamma-T), tocotrienols (T3s) and aspirin reduce oxidative stress and ROS overproduction (up arrow).

INTRODUCTION

Colorectal cancer (CRC) remains a major contributor to cancer cancer worldwide and accounts for about 9% of overall cancer incidence[1]. There is, however, a quite remarkable country-to-country variation and CRC appears to be primarily a disease of Western life-style[1]. In India the incidence of CRC is about one seventh that of the United States[2]. Moreover, immigrants from low-risk countries who move to high-risk countries generally assume the high-risk profile of the new host country[1]. These data suggest that environmental factors could be important in the etiology of sporadic CRC and provide some hope that chemopreventive/lifestyle strategies could have a significant impact on reducing CRC incidence. The primary purpose of this review is to evaluate the potential role that pro-oxidant and antioxidant factors play in the development of CRC. The roles of these factors are of major importance to the advice that oncologists provide to cancer patients and that nutritionists provide to the general population. The food industry also has a vested interest in these issues since many foods are labeled as being "high in antioxidants" with the implicit promise of promoting health.

PRO-OXIDANTS AND ANTIOXIDANTS IN CRC

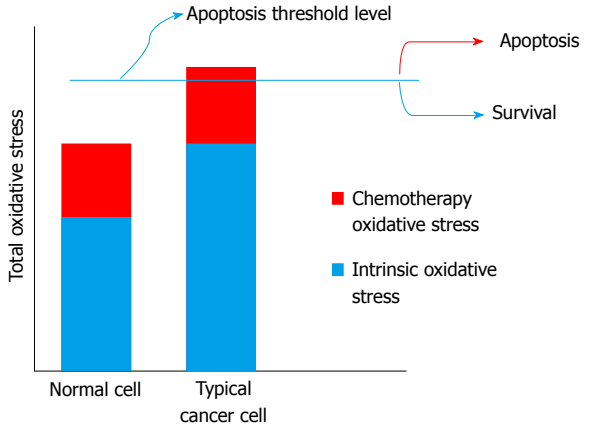

Figure 2 Conceptual framework for the selective action of chemotherapeutic agents that induce oxidative stress. Many cancer cells show a high level of intrinsic oxidative stress ("blue" component of total oxidative stress) compared to normal cells. Chemotherapeutic agents often act by inducing an additional level of oxidative stress ("red" component of total oxidative stress) that is sufficient to reach an apoptotic threshold (blue line) in a typical cancer cell but not a normal cell.

The role of both antioxidants and pro-oxidants in colon cancer has become a topic of intense interest and controversy[3-6]. A great body of evidence supports the view that *in vivo* oxidative stress and the accompanying reactive oxygen species (ROS) are genotoxic and contribute to the development of colon cancer and cancers in general (Figure 1)[7]. ROS are thought to be a major source of endogenous DNA damage and at least one hundred oxidative modifications to DNA have been identified[8,9]. It is plausible to assert, therefore, that antioxidants could be beneficial by minimizing the genotoxic insults caused by ROS and thereby reduce the incidence of cancers. In this capacity, antioxidants in food or in dietary supplements would be acting as long-term chemopreventive agents. Moreover, it is now well recognized that many cancer cells exhibit an enhanced level of intrinsic oxidative stress that plays a causative role in the expression of many oncogenic phenotypes[10,11]. The ROS giving rise to the enhanced level of intrinsic oxidative stress in cancer cells are thought to promote oncogenic phenotypes by virtue of their roles in modulating redox sensitive signal transduction mechanisms[12]. It follows, that *in vivo* antioxidant agents (chemical and enzymatic) that lower ROS could potentially inhibit the expression of aggressive cancer phenotypes. Antioxidants could, therefore, be chemopreventive by reducing both genotoxicity and by slowing cancer progression.

DO ANTIOXIDANTS INTERFERE WITH CHEMOTHERAPY?

On the other hand, many effective pro-oxidant chemotherapeutic agents rely on inducing additional oxidative stress in cancer cells, thereby driving them into apoptotic cell death (Figure 2). This process is thought to have some degree of selectivity since cells with a "normal" level of oxidative stress would not be sufficiently stressed by pro-oxidant chemotherapeutic agents to reach the

threshold at which apoptosis would be triggered. A key concern, however, is that cancer patients with a high level of dietary/supplement antioxidant intake could be resistant to pro-oxidant chemotherapeutic agents[3,6]. It is likewise plausible that a high level of antioxidant intake could shield normal tissues from the cytotoxic effects of pro-oxidant chemotherapeutic agents, thereby reducing many of the severe side effects associated with these agents. These issues were expertly reviewed in 2008 with the conclusion that supplemental antioxidants should be avoided during chemotherapy and radiation therapy based on their potential for protecting tumors and reducing the effectiveness of the pro-oxidant therapies. Little has changed from 2008 and this assessment remains valid. Nevertheless, this remains an area where more evidence-based medicine is needed. As is often the case in clinical research, a more nuanced approach is required that is cancer specific, dose and time controlled and focused on specific antioxidants.

A SYSTEMS BIOLOGY APPROACH TO REDOX ISSUES IN CRC

The complex set of interconnected events that give rise to CRC are unlikely to be fully replicated (or even well elucidated) in cell-based studies, animal models, observational studies or even short term clinical intervention trials. A systems biology approach in which an organism is considered a dynamic set of interacting organs, tissues, cells and molecular level components is more realistic, especially since time-dependency and interconnecting environmental factors are also key to this approach[13].

OXIDATIVE STRESS AND THE INTESTINAL MICROBIOME

The utility of a systems biology approach to oxidative stress and CRC is firmly illustrated when considering the role of the intestinal microbiome. The intestinal microbiome is the community of commensal, symbiotic, and pathogenic microorganisms sharing the space within the intestinal lumen. CRC, in most cases, arises from the epithelial cells of the large intestine, which have suffered DNA damage and a subsequent loss of epithelial differentiation towards a phenotypic expression closer to that of mesenchymal cells (the epithelial-to-mesenchymal transition or EMT)[14]. The EMT of DNA-damaged colonic epithelial cells is thought to promote metastasis to other essential organs. The small intestine, despite its name, has an epithelial surface area some 30 times larger than the large intestine. This large difference is due to the fact that the small intestine, unlike the large intestine, is very convoluted and has villi. Nevertheless, cancers of the small intestine are rare, amounting to only 2.3% of cancers of digestive system in the United States[15]. A complete understanding of this differential rate of cancer incidence along the digestive track is lacking but accumulating data suggest that oxidative stress could be an important factor.

A major difference between the small intestine and large intestine is the number of bacteria present. The duodenum and jejunum (66% of the small intestine) contain low numbers of bacteria but this number markedly increases by about four orders of magnitude in the distal ileum and in the large intestine[16]. Owen *et al*[17] have found that the human fecal matrix is capable of generating abundant ROS. In marked contrast, many isolated and cultured aerobic or anaerobic fecal bacteria do not generate abundant ROS[17]. Babbs *et al*[18] also found that fecal matrix generates a high flux of ROS being the equivalent of 10000 rads of gamma irradiation per day. This high level of ROS production stopped after autoclaving the feces suggesting the direct involvement of fecal bacteria. The *in vitro* experiments of both Owen *et al*[17] and Babbs *et al*[18] must be interpreted with some caution and future work in this area is needed given the importance of understanding the role of digestive system microflora in cancer development. Nevertheless, these studies suggest that the interaction between fecal bacteria and the fecal matrix generates oxidative stress.

IS COLONIC OXIDATIVE STRESS SUFFICIENT TO CAUSE GENOTOXIC DAMAGE?

Whether or not colonic oxidative stress arising from colonic bacteria is capable of causing significant *in vivo* genotoxicity in humans is not yet known. The *in vitro* results of Wang and Huycke[19] are, however, very relevant in this regard. These investigators found that *Enterococcus faecalis* (*E. faecalis*), a prevalent fecal bacteria that uniquely generates extracellular superoxide, is quite effective at promoting chromosomal instability (CIN) in mammalian cells at a level equal to that 2 gray (or 200 rad) of gamma-irradiation. *E. faecalis* is thought to generate superoxide by virtue of a rudimentary respiratory chain in which an electron is transferred to oxygen by membrane-associated demethylmenaquinone[19].

Under normal circumstances, fecal bacteria do not come into contact with the epithelial cells of the large intestine, which is covered by layers of dense mucin (10-90 microns thick). With this fact in mind, Wang and Huyche[19] reasoned that M cells (or microfold cells) in the colon could potentially transport *E. faecalis* across the intestinal lumen to macrophage cells (antigen presenting cells) across the epithelial barrier (into the subepithelial dome) for immunological processing (*i.e.*, the innate immune system). This is schematically illustrated in Figure 3. Accordingly, these investigators tested the hypothesis (*in vitro*) that macrophages that have phagocytized *E. faecalis* could generate diffusible oxidation products that could induce CIN in surrounding hybrid hamster cells containing human chromosome 11. Their results were consistent with this hypothesis: a 2.5 fold increase in CIN was found in hamster cells co-incubated macrophages that phagocytized *E. faecalis* compared to hamster cells co-

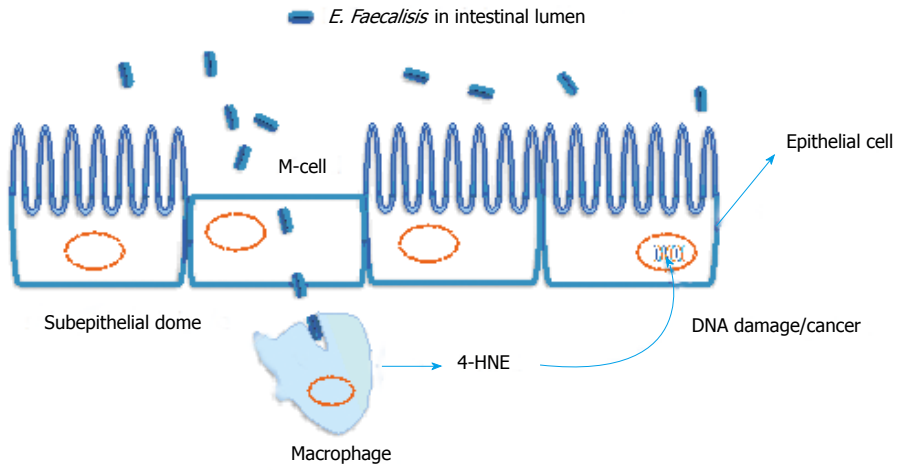

Figure 3 Role of pro-oxidant intestinal bacteria in colorectal cancer. *Enterococcus faecalis* (*E. faecalis*) an intestinal bacteria with the unique ability to generate superoxide radicals. Intestinal microfold cells (M-cells) may transport *E. faecalis* from the intestinal lumen to macrophages in the subepithelial dome where macrophage cyclooxygenase-2 (COX-2) and lipid peroxidation can generate 4-hydroxynonenal (4-HNE), which promotes DNA damage/chromosomal instability to nearby epithelial cells[20].

incubated with macrophages not having phagocytized *E. faecalis*. Control experiments using *Escherichia coli*, which generates only low levels of superoxide, elicited only a modest degree of mammalian cell CIN in their model.

GAMMA-TOCOPHEROL BUT NOT ALPHA-TOCOPHEROL INHIBITS CHROMOSOMAL INSTABILITY IN MAMMALIAN CELLS INDUCED BY *ENTEROCOCCUS FAECALIS*

Quite interestingly, Wang and Huyche[19] found that 200 μmol gamma-tocopherol, but not alpha-tocopherol, was able to completely inhibit the CIN inflicted on hamster cells when co-incubated with macrophages having phagocytized *E. faecalis*. Moreover, cyclooxygenase-2 (COX-2) overexpression was found in the macrophages having phagocytized *E faecalis* and COX-2 inhibitors (as well as superoxide dismutase) blocked the induced CIN in hamster cells.

In a logical follow-up to this *in vitro* work, these investigators provided convincing evidence that the diffusible product of oxidative stress induced by *E. faecalis* was 4-hydroxy-2-nonenal (4-HNE), which is a well-known aldehyde by-product arising from the lipid peroxidation of omega-6 polyunsaturated fatty acids (*e.g.*, arachidonic acid)[20] (Figure 4). As mentioned below, arachidonic acid is a proinflammatory dietary fatty acid and dietary arachidonic acid is a risk factor for CRC. Moreover, silencing glutathione-S-transferase alpha4 (GST-alpha4) in colonic epithelial cells increased their susceptibility to 4-HNE CIN. GST-alpha4 detoxifies 4-HNE by covalently complexing it with GSH. Similarly, silencing COX-2 decreased 4-HNE production by *E. faecalis* infected macrophages and depleting GSH (the primary intracellular antioxidant) increased 4-HNE production[21].

In an outstanding pre-clinical *in vivo* experiment, these investigators also found that interleukin-10 knockout mice (IL-10$^{-/-}$) colonized with superoxide producing *E. faecalis* developed both inflammation and CRC[20]. IL-10$^{-/-}$ mice develop colitis and are a model for human inflammatory bowel disease. In a parallel experiment with a strain of *E. faecalis* that does not produce superoxide (delta-men) the IL-10$^{-/-}$ mice showed inflammation but not CRC[20]. Unfortunately, the investigators did not determine if supplementation with gamma-tocopherol prevented CRC in the animal model using the superoxide producing *E. faecalis*. The overall model proposed by these investigators is provided in Figure 3. Collectively, this work provides a very compelling hypothesis for the etiological factors promoting CRC, *i.e.*, M-cells transport pro-oxidant intestinal bacteria to macrophages in the subepithelial dome and through the action of macrophage COX-2 and lipid peroxidation on pro-inflammatory n-6 n-6 polyunsaturated fatty acid (PUFA) generate 4-HNE which diffuses to nearby epithelial cells inducing genotoxicity eventually resulting in CRC. This is a powerful model and does much to explain the interconnections between the known risk factors for CRC and their relationship to the oxidative stress (Figure 1).

DYSFUNCTION OF THE INTESTINAL MICROBIOME, INFLAMMATORY BOWL DISEASE AND OXIDATIVE STRESS

It is becoming increasingly clear that dysfunction of the intestinal microbiome is very much related to inflammatory bowl disease(s) such as Crohn's disease and ulcerative colitis. Inflammatory bowel diseases (IBDs) are known to increase the risk of CRC and inflammation (in general) is also accompanied by increased oxidative stress[20]. With rapid technical advances it will soon be routinely possible for individuals to have their exomes sequenced in an effort to identify markers for cancer susceptibility. For most types of cancer, exome sequencing alone should be sufficient but CRC might be an exception. The collective

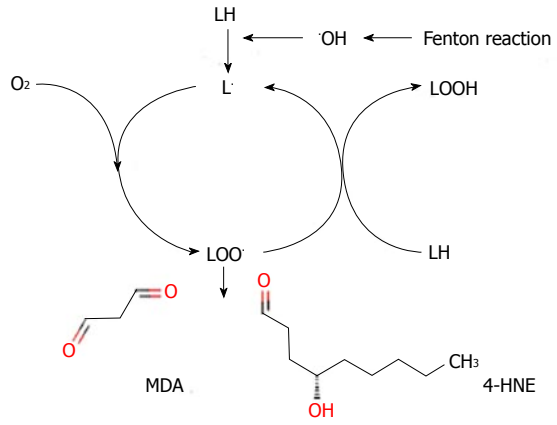

Figure 4 Lipid peroxidation and mutagens. Lipids containing PUFA (LH) are oxidized to form damaging lipid peroxy radicals (LOO·). These radicals can generate mutagenic aldehydes such as malondialdehyde (MDA) and 4-hydroxy-2-nonenal (4-HNE) from n-6 PUFAs. Lipid peroxidation can be initiated by the formation of highly reactive hydroxyl radicals (·OH) arising from the Fenton reaction.

genomes of the intestinal microbiome is estimated to be about 100 times that of the human genome and is of direct significance to CRC. Although beyond the scope of this review, it is now clear that that there are complex interactions between the intestinal microbiome, IBD and metabolic processes in the large intestine[22]. Of particular interest, however, is the model proposed by Morgan *et al*[22] which suggests that IBD could be accompanied by a shift in the normal microbiome to microbes utilizing mucin as a primary energy source and thereby compromise the barrier function of mucin in protecting colonic epithelial cells from contact with microbes. Mucin is rich in the sulfur-containing amino acids needed to synthesize glutathione (GSH), which is a key intracellular antioxidant. The loss (or thinning) of the mucin barrier would, in turn, result in increased inflammation and oxidative stress that could then select for those microbes efficient at using GSH to survive in an oxidatively stressed environment.

THE GENETIC CHARACTERISTICS OF CRC AND OXIDATIVE STRESS

The types of mutations that occur in CRC are of interest since this information is relevant to etiological factors and their origin. The lung, the esophagus and the colon, unlike most other internal organs, are directly exposed to a wide variety of environmental mutagens and, not unexpectedly, have a large number of non-synonymous mutations per tumor compared with other adult solid tumors[23]. Nonsynonymous mutations are those that alter the encoded sequence of a protein. Lung cancer is thought to be largely (about 90%) due to mutagens in cigarette smoke. Cigarette smoking is a major oxidative stressor and it is reasonable to suggest that smoke derived carcinogens and oxidants in tobacco tar could make their way to the colon.

OXIDATIVE STRESS CAN INACTIVATE THE DNA MISMATCH REPAIR MECHANISM SYSTEM IN CRC

CRC mutations fall into two general categories: (1) those with mutations accompanied by microsatellite instability (MSI) in which there is a defective DNA mismatch repair (MMR) mechanism during DNA replication (about 15% of all CRC); (2) those with mutations accompanied by microsatellite stability (MSS) but with chromosomal instability[23]. Microsatellites are repeating sequences of 2-6 base pairs of DNA.

MSI CRC is associated with a very large number of non-synonymous mutations yet has a better prognosis than MSS CRC[23,24]. A high frequency form of MSI (MSI-H), where over 40% of the assayed microsatellites are mutated, is associated with germline mutations in the protein complexes forming the MMR system or with epigenetic silencing of MMR protein expression by DNA hypermethylation[25]. A second, low frequency form of MSI (MSI-L) also occurs in which less than 20% of the assayed microsatellites are mutated[25]. Quite curiously, 10%-20% of sporadic CRC of the MSI-L variety show no evidence of mutations in MMR proteins and are only rarely associated with epigenetic silencing of MMR protein expression by DNA hypermethylation[25]. MSL-L is, however, found in CRC associated with ulcerative colitis, a long lasting inflammatory bowel disease with well-documented evidence of oxidative stress[25,26]. Chang *et al*[25] have shown that oxidative stress can inactivate the MMR system leading to the suggestion that this mechanism could be responsible for the MSI-L seen in CRC associated with chronic inflammation such as ulcerative colitis and/or smoking and/or high alcohol consumption which are all oxidative stressor factors (see below).

WHAT DO OBSERVATIONAL STUDIES TELL US ABOUT THE INCIDENCE OF CRC AND ENVIRONMENTAL ANTIOXIDANTS AND PRO-OXIDANT FACTORS?

Observational studies are fraught with limitations and do not directly speak to causality. Nevertheless, such studies are often all that is available and have the advantage that very large subject populations can be studied over very long time periods at a reasonable cost. Observational studies are very useful for hypothesis building, particularly when combined with pre-clinical data from cell and animal models. The genetic data, summarized above, suggests that environmental mutagens contribute to CRC. As detailed below, a strong case can be made that many of the environmental factors known to contribute to CRC incidence are also sources of oxidative stress and genotoxicity.

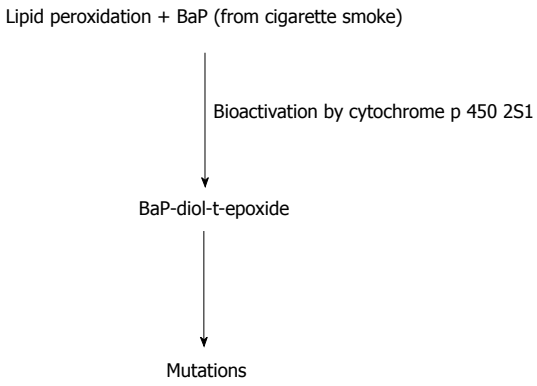

Figure 5 Lipid peroxidation, smoking and the activation of benzo[a]pyrene. Lipid peroxidation and cytochrome P450 2S1 (CYP2S1) can activate benzo[a]pyrene (BaP) to the potent mutagen, benzo[a]pyrene-r-7,t-8-dihydrodiol-t-9,10-epoxide (BaP-diol-t-epoxide). A high expression of CYP2S1 is associated with colorectal cancer and a poor prognosis (see text).

CIGARETTE SMOKE AS AN ENVIRONMENTAL SOURCE OF OXIDATIVE STRESS AND GAMMA-TOCOPHEROL AS A DIETARY ANTIOXIDANT

Cigarette smoking and is thought to contribute to about 12% CRC incidence[1]. For reasons that are not clear, the increased risk of CRC due to smoking appears to be greater in women than in men[27]. Smoking is a major source of free radicals in both the gaseous and tar phases[28,29] and is a major contributor to *in vivo* oxidative stress. Overwhelming evidence shows that smoking increases many systemic biomarkers for oxidative stress such as breath pentane[30], plasma protein carbonyls[31], F2 isoprostanes[32] and also causes an increased vitamin E consumption through an oxidative stress pathway[33-35]. Cigarette smoke contains a significant amount of reactive nitrogen species (RNS) as well as ROS[36]. Gamma-tocopherol (the main dietary form of vitamin E) has a unique ability to react with RNS to form 5-nitro-gamma-tocopherol (NGT) and levels of NGT are about two fold higher in smokers compared to nonsmokers[36]. We do not yet know if supplementation with gamma-tocopherol would reduce the genotoxic effects of RNS cigarette smoke and thereby reduce CRC or cancer in general.

CIGARETTE SMOKE, MUTAGENIC POLYCYCLIC AROMATIC HYDROCARBONS AND LIPID PEROXIDATION

Cigarette smoke (and the high temperature cooking of meat) is also a source of polycyclic aromatic hydrocarbons (PAHs), which are subsequently activated to potent mutagens. A recent case-cohort study suggests that a 57% increase in CCR is associated with a doubling of the level of aromatic DNA adducts[37]. Benzo[a]pyrene (BaP) is a major and potent PAH carcinogen found in cooked meats and tobacco smoke. It has long been known that the activation of BaP to its ultimate carcinogen can be promoted by the process of lipid peroxidation[38]. DNA adducts of carcinogenic BaP metabolites have been found in human colon mucosa[39].

Recent work now suggests (Figure 5) that lipid hydroperoxides support the cytochrome P450 mediated activation of benzo[a]pyrene-trans-7,8-dihydrodiol (BaP-7,8-diol) into the highly mutagenic the highly mutagenic and carcinogenic benzo[a]pyrene-r-7,t-8-dihydrodiol-t-9,10-epoxide (BaP-diol-t-epoxide)[40]. Cytochrome P450s (CYPs) are a superfamily of enzymes that catalyze the oxidation of xenobiotic organic substances such PAHs as well as a variety of endogenous compounds. CYP2S1 is the particular cytochrome P450 that effectively activates BaP-7,8-diol[40]. It is very relevant, therefore, that the P450 profile of CRC has been determined and CRC tissues show a higher level of CYP2S1 expression compared to normal CR tissue[41]. Moreover, a higher CRC expression of CYP2S1 was associated with poor prognosis[41]. Collectively, the above suggests that smoking induced oxidative stress, and increased exposure to BaP, could result in lipid peroxidation driven production of carcinogenic BaP-diol-t-epoxide with increased CCR incidence and poor prognosis.

DIETARY ARACHIDONIC ACID INTAKE AS A PRO-OXIDANT STRESSOR

Some observational studies suggest that the dietary consumption of arachidonic acid, a proinflammatory and pro-oxidant (as detailed below) PUFA is associated with an increased risk of CRC[42,43]. In contrast n-3 PUFAs (primarily from marine sources) are generally considered to be anti-inflammatory and have anti-neoplastic properties, at least in animal and human cell models[44]. A recent large-scale study of over 73000 Chinese women suggests that the ratio of dietary total n-6 to n-3 PUFA ratio is strongly associated with the incidence of CRC: compared to women in the lowest quintile, the relative risk of CRC was 1.95 for women in the highest total n-6 to n-3 PUFA quintile[44].

COX-2 OVEREXPRESSION, OXIDATIVE STRESS AND CRC

The cyclooxygenase enzymes catalyze the rate-limiting step of prostaglandin formation from arachidonic acid. There are two known isoforms of cyclooxygenase: cyclooxygenase-1 (COX-1) and COX-2. COX-1 has a constitutive promoter and is expressed in many tissues to maintain normal physiological functions such as the maintenance of renal blood flow, gastric mucosa, and platelet aggregation. COX-2 has an inducible promoter that contains a number of active regulatory elements including: FOXM1, cyclic AMP response element bind-

ing protein (CREB), NFkappaB, AP-1, p53, and PPAR gamma[45,46]. COX-2 overexpression is strongly associated with a number of cancers[47]. A large amount of evidence supports the view that a constitutive expression of the COX-2 enzyme is a contributing factor in the promotion of colon carcinogenesis as well as other cancers[48,49]. COX-2 overexpression is an unfavorable prognostic factor for numerous cancers including CRC[47-49] while silencing COX-2 reduces the tumorigenesis of CRC as well as the metastatic potential of CRC and other cancers[50,51]. COX-2 activation can occur through numerous signals, which contribute to the fact that the mechanism behind COX-2 activation has not been fully elucidated. Inflammation, viral and bacterial infections, phorbol esters, lipopolysaccharides, transforming growth factor beta, UVB exposure, gamma-irradiation, and mechanical shear stress can all be responsible for the activation of COX-2[52,53].

In HT-29 human colon cancer cells nontoxic doses of hydrogen peroxide (10 μmol) results in cancer cell proliferation, but toxic doses of 1000 μmol induce apoptosis[54]. The stimulation of cell proliferation was accompanied by an increase in COX-2 and apoptosis from the high-dose hydrogen peroxide was negatively correlated with COX-2 expression[54]. These data suggests that the balance of proliferation and apoptosis of cancer cells is dependent upon the concentration of ROS and can be correlated with COX-2 expression[54].

However, the roles COX-2 activation and suppression have on pro-oxidants, ROS, and antioxidants in carcinogenesis are not clear. In some studies COX-2 expression has led to increased oxidative stress, while in other studies increased oxidative stress has occurred through the inhibition of COX-2. For example, COX-2 mediated arachidonic acid metabolism was identified as a potential source of ROS in human intestinal epithelial cells (FHs 74 Int) exposed to monohaloacetic acids[55]. Viral induction of COX-2 has led to increased oxidative stress[56]. In a study examining the effects of nitric oxide-releasing non-steroidal antin flammatory drugs (NO-NSAIDs), treatment of human colon cancer cells lines with NO-NSAIDs produced a cytotoxic effect in all cell lines tested and an increased COX-2 activity was observed with concomitant oxidative stress[57].

There are examples of chemotherapy agents that enhance the expression of COX-2. For example, oral mucosal staining following cytotoxic chemotherapy (with various chemotherapeutic regimens including: doxorubicin/docetaxel/cyclophosphamide/methotrexate/5-fluorouracil; cyclophosphamide/methotrexate/5-fluorouracil; docetaxel alone; 5FU/folinic acid; and 5FU/leucovorin) demonstrate an increase COX-2 expression[58]. Colorectal tissues from patients treated with preoperative radiotherapy demonstrated increased expression of COX-2[59]. While the above data suggests that expression of COX-2 induces oxidative stress, there is a balanced amount of evidence showing that inhibition of COX-2 induces oxidative stress. For example, inhibition of aldose reductase (AR), an enzyme that catalyzes the reduction of lipid aldehydes and their glutathione conjugates, results in a growth reduction of human colon cancer cell through the inhibition of TNF-alpha induced activation of PKC and NFkappaB, which results in the abrogation of COX-2 mRNA and protein expression[60]. AR inhibition results in suppression of oxidative stress in inflammatory disorders[61]. Further, inhibition of phorbol ester-mediated induction of COX-2 in colon carcinoma cells by 15-deoxy-delta(12,14)-prostaglandin J(2) (15d-PGJ(2)) results in intracellular oxidative stress through the inhibition of AP-1 activation[62].

The chemical treatment of animals with azoxymethane (AOM) is a commonly accepted model for carcinogenesis, which results in the formation of aberrant crypt foci (ACF), precursor lesions to colon cancer. Pterostilbene (PS) had been reported to prevent chemical-induced colon carcinogenesis by anti-inflammatory and pro-apoptotic properties[63]. In a study examining the effects of PS on AOM-induced colon tumorigenesis, it was discovered that PS reduced AOM-induced tumor formation, ACF, as well as lymphoid nodules. In addition, PS treatment resulted in reducing the expression of oxidative inflammatory markers NFkappaB, inducible nitric oxide synthase, COX-2 and AR, while enhancing the expression of antioxidant enzymes such as hemeoxygenase-1 and glutathione reductase *via* NF-E2 related factor 2 signaling[63]. While it is not clear what conditions lead to oxidative stress through COX-2 signaling, these data suggest that the role of COX-2 in carcinogenesis is correlated with antioxidant signaling/pro-oxidant signaling and that more investigation is needed to understand these mechanisms in CRC.

HEAVY ALCOHOL CONSUMPTION AS A PRO-OXIDANT STRESSOR

Heavy alcohol consumption has been linked to an increase CRC risk as well well as an increased incidence of tumors in the distal colon[1]. Individuals with a family history of CRC are particularly at risk, with a relative risk of 2.8 compared to nondrinking individuals with no family history of CRC[64]. Similarly, patients with chronic alcohol dependence also show an increased level of oxidative stress biomarkers such as plasma protein carbonyl levels[65]. Alcohol is, however, likely to be procarcinogenic by multiple mechanisms. Alcohol is metabolized to acetaldehyde, which is a highly toxic mutagen causing point mutations[66,67]. Alcohol metabolized by the cytochrome P450 system of the endoplasmic reticulum leads to the production of both acetaldehyde and ROS[67]. ROS can directly cause DNA damage and can also lead to increased lipid peroxidation with the generation of genotoxic aldehydes, *e.g.*, MDA and 4-HNE.

ANTIOXIDANT ENVIRONMENTAL FACTORS

Fruit and vegetables

Fruit and vegetables have a relatively high content of

antioxidant compounds and many observational studies have shown that their frequent consumption is associated with a decreased risk of CRC. Nevertheless, a very large scale and well-conducted study in 2000 found "high consumption of fruit and vegetables did not appear to be protective against cancers of the colon and rectum in our large United States cohorts"[68]. Recent results from the Shanghai Men's Health Study showed that the consumption of fruits but not vegetables was associated with a reduced risk of CRC in middle-aged and older Chinese men[69]. In the United States, folate added to many common foods items and present in most multivitamins may be preventing colon cancer and negating the need to get this vitamin from fruits and vegetables[68].

The Iowa Women's health study (35000 women) found that a high intake of vitamin E was associated with a reduced risk of CRC[70]. Most of the vitamin E intake in this study was from multivitamin supplements and the form of vitamin E (see below) was not specified. Moreover, during the time period (1986-1990) for this study, most multivitamin supplements had very high levels of iron which is a quite potent pro-oxidant that can promote lipid peroxidation[71].

ASPIRIN, AN ANTIOXIDANT CHEMOPREVENTIVE FACTOR

It has long been known that the daily consumption of aspirin is associated with a significant decrease in CRC risk. Aspirin is a direct quencher of the genotoxic hydroxyl radical and the formation of hydroxylated aspirin derivatives (2,3- and 2,5-dihydroxybenzoic acid) has long been utilized as very sensitive *in vivo* biomarker for oxidative stress[72]. Most relevant to this review is the well-documented ability of aspirin to inhibit COX-1 and COX-2[73]. Data from two large studies now suggests that aspirin's protective effect can be modified by the BRAF gene[74] which codes for a protein called serine/threonine-protein kinase B-Raf (a member of the Raf kinase family). Constitutive activation of the Ras-mitogen-activated protein kinase (MAPK) kinase pathway is of major importance in CRC and this can occur by oncogenic mutations in BRAF that upregulate the Ras-MAPK kinase pathway resulting (among many other important cancer related events) in an overexpression of COX-2[75]. Nishihara *et al*[74] found that aspirin use reduced the risk of CRC in individuals with wild type BRAF but not in individuals with oncogenic mutated BRAF. As also mentioned above, these data emphasize the potential interconnections between COX-2, CRC and oxidative stress.

LARGE SCALE CLINICAL TRIALS OF VITAMIN E AND CANCER PREVENTION-THE MANY DETAILS AND THE MANY DEVILS

The Selenium and Vitamin E Cancer Prevention Trial (SELECT) was a $130 million trial that concluded that "vitamin E" did not prevent prostate cancer, colon cancer, or any other cancer[76]. In a more ominous note, a follow up to the SELECT trial concluded that "Dietary supplementation with vitamin E significantly increased the risk of prostate cancer among healthy men"[77]. Vitamin E is the major *in vivo* lipid soluble antioxidant and it quenches the lipid peroxyl radicals that propagate during lipid peroxidation (*c.f.*[78]). As is often the case in biomedical research, there are many "devils" in the details of the SELECT study that are worthy of notice. An often-overlooked detail lies in the particular form of vitamin E used in the study. For the SELECT trial this was all-racemic-alpha-tocopheryl acetate at a dose of 400 IU/d taken over a period of about 5.5 years in men who were 50-55 or older at the start of the study. As it happens, "vitamin E" is not a single-organic compound and there are at least eight natural forms, *i.e.*, alpha-, beta-, gamma- and delta-tocopherols as well as the corresponding four tocotrienols (*c.f.*[79,80]). To make matters more complicated, vitamin E isoforms have asymmetric carbons where each such carbon is attached to four different groups of other atoms. Tocopherols, for example, have three asymmetric carbons, each of which could have an R- or S-stereo-configuration.

All naturally occurring forms of tocopherols have the R-stereo-configuration. The form of vitamin E used in the SELECT study was "synthetic" where the configuration at each of the asymmetric carbons is an equimolar mixture of both the R- and S-stereoisomers (at each of the three asymmetric carbons): this is the form of vitamin E found in most commercial supplements although it is sometimes mistakenly labeled dl-alpha-tocopheryl acetate. All-racemic alpha-tocopheryl acetate is an equimolar mixture of eight stereoisomers with only one eighth of which is the naturally occurring RRR-alpha-tocopherol. The other seven stereoisomers are essentially xenobiotics whose detailed biochemical properties (and potential modulation of signal transduction pathways) are largely unstudied. Moreover, the bioavailability of all-racemic-alpha-tocopheryl acetate is about half that of RRR-alpha-tocopheryl acetate. Nevertheless, the ability of R- or S-vitamin E isomers to quench free radicals (lipid peroxyl radicals) and prevent lipid peroxidation is very similar.

ARE DIETARY ANTIOXIDANTS USELESS AS CHEMOPREVENTIVE AGENTS?

The SELECT study certainly suggests, that in healthy middle aged men, taking a potent lipid soluble antioxidant for half a decade or more did nothing to prevent prostate cancer, colon cancer or any other cancer diagnosed in this study. Does this mean that dietary antioxidants are useless as chemoprevention agents? Using a strictly evidenced based approach the answer is "we cannot be sure." CRC is very much a disease of aging with the incidence markedly increasing after the age of 50. This

suggests that many of the driver mutations responsible for CRC have already accumulated by mid-age. For any antioxidant to be effective as a chemopreventive agent that blocks free radical mediate genotoxicity it is reasonable to suggest that it must be consumed at an effective level starting at an early age. By mid-life too many oncogenic driver mutations may already be in play and a five-year period may also be too short. It may also be that the particular chemical form of the antioxidant is of critical importance since this is likely to play a role: (1) in its bioavailability; (2) what ROS/RNS are being modulated; (3) what organs/tissues is the antioxidant being delivered to; (4) the cellular and subcellular distribution of the antioxidant; and (5) does the antioxidant have any other relevant anticancer properties unrelated to its ability to function as an antioxidant (*e.g.*, modulate carcinogenic signal transduction pathways).

WAS THE RIGHT FORM OF VITAMIN E USED IN THE SELECT TRAIL?

Neither tissue culture experiments nor animal models support of a strong anti-cancer role for all-racemic-alpha tocopherol or RRR-alpha-tocopherol. In contrast, both gamma-tocopherol[81,82] and tocotrienols[83] have anticancer effects that are now well documented. Gamma-tocopherol is the primary form of vitamin E in the American diet. It is quite interesting that the anticancer effects of tocotrienols are attenuated by supplementation with alpha-tocopherol[84]. Moreover, supplementation with alpha-tocopherol lowers plasma levels of gamma-tocopherol.

In addition to issues related to vitamin E stereochemistry, it should be noted that the form used in the SELECT study was a vitamin E ester ("yl") rather than the free form ("ol"). Any vitamin E ester, such as alpha-tocopheryl acetate, is not an antioxidant since the functional phenol group is blocked by esterification. An esterase must act on the vitamin E before it is converted into an active "ol" antioxidant with the ability to quench lipid peroxyl radicals. In humans, vitamin E esters can have half the bioavailability compared to the corresponding free or unesterified form[85]. Much of the vitamin E in a high dose gel capsule (*e.g.*, 200 IU) is not absorbed and is found in feces. It may well be that free tocopherol in the fecal matrix could reduce oxidative stress in the colon whereas tocopheryl esters would have no such effect. Quite surprisingly, some vitamin E esters (but not alpha-tocopheryl acetate) have anticancer effects that are not shared by the unesterified (free) alpha-tocopherol. Vitamin E succinate (or more precisely alpha tocopheryl succinate) for example has been found to be effective in reducing CRC in a mouse xenograft model[86,87].

The non-alpha-tocopherol forms of vitamin E have not yet been tested in large scale randomized, placebo-controlled studies. In any event, it is not biochemically justified to make clinical conclusions about all forms of vitamin E (or antioxidants in general) based on the results from a single form (such as all-rac-alpha-tocopheryl acetate) as was done in the SELECT study and in much of the "web" literature.

CONCLUSION

The evidence presented in this review presents a compelling case supporting the view that environmental oxidative stressors are causally related to the development of CRC. Moreover, the molecular and cellular details whereby oxidative environmental stress is translated into genotoxic damage to the epithelial cells of the large intestine are becoming increasingly clear as detailed above. The intestinal microbiome can be a source of oxidative environmental stress and can, *via* intestinal M-cells, transmit oxidative stress to macrophages in the subepithelial dome where subsequent genotoxic damage to colonic epithelial cells is likely.

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Xenoestrogens challenge 17β-estradiol protective effects in colon cancer

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Abstract

Several epidemiological, cellular, and molecular studies demonstrate the role of environmental chemicals with endocrine disrupting activities, typical of Westernized societies, in the pathogenesis of numerous diseases including cancer. Nonetheless this information, the design and execution of studies on endocrine disruptors are not yet cognizant that the specific actions of individual hormones often change with development and ageing, they may be different in males and females and may be mediated by different receptors isoforms expressed in different tissues or at different life stages. These statements are particularly true when assessing the hazard of endocrine disruptors against 17β-estradiol (E2) actions in that this hormone is crucial determinant of sex-related differences in anatomical, physiological, and behavioral traits which characterize male and female physiology. Moreover, E2 is also involved in carcinogenesis. The oncogenic effects of E2 have been investigated extensively in breast and ovarian cancers where hormone-receptor modulators are now an integral part of targeted treatment. Little is known about the E2 preventive signalling in colorectal cancer, although this disease is more common in men than women, the difference being more striking amongst pre-menopausal women and age-matched men. This review aims to dissect the role and action mechanisms of E2 in colorectal cancer evaluating the ability of estrogen disruptors (*i.e.*, xenoestrogens) in impair these E2 actions. Data discussed here lead to define the possible role of xenoestrogens in the impairment and/or activation of E2 signals important for colorectal cancer prevention.

Key words: 17β-Estradiol; Estrogen receptors; Xenoestrogens; Bisphenol A; Flavonoids; Colorectal cancer

Core tip: In this review, we will report recent data, including ours, on 17β-estradiol (E2) action in colon health and disease discussing on how environmental chemicals with endocrine disrupting activities could impact on these E2 effects in colon cancer. In particular, two plant-derived flavonoids (*i.e.*, naringenin, Nar, and quercetin, Que) and one synthetic food-contaminant bisphenol A will be reported as prototype molecules to evaluate how xenoestrogens can impact on cell proliferation/apoptosis balance, the critical physiological function of E2 in colon.

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INTRODUCTION

Since many years it was believed that the primary function of 17β-estradiol (E2) was in the development of female secondary sexual characteristics and subsequent regulation of reproductive function. However, this has been recognised as an over-simplification and now it is well known that this sex steroid hormone elicits a myriad of biological responses directed towards profoundly changing male and female physiology[1]. As a consequence, it is not surprising that E2 is also involved in

diseases including carcinogenesis. The oncogenic effects of E2 have been investigated extensively in breast[2] and ovarian[3] cancers where hormone-receptor modulators are now an integral part of targeted treatment. Little is known about the E2 preventive signalling in colorectal cancer although women are less susceptible to this cancer than men[4].

Both physiological and the contradictory pathologic actions of E2 are mediated by two receptor subtypes (*i.e.*, ERα and ERβ) members of the nuclear receptor superfamily which are defined as ligand-activated transcriptional factors. ERα and ERβ are localized in the cytoplasm and in the nucleus of E2-target cells where they are associated, in the resting state, to heat shock proteins. A small pool of these receptors is palmitoylated and localized at the plasma membrane in association with caveolin-1[5]. E2 binding to the cytosolic ER population (both ERα and ERβ) induces conformational changes that facilitate ER homo/heterodimerization, nuclear translocation, and binding to specific DNA recognition sequences (*i.e.*, estrogen responsive elements; ERE)[1]. In this classical/genomic mode of action, ERα and ERβ promote E2-sensitive gene transcription, ERβ being approximately 30% less efficient than ERα[6]. It is well established that the main role of the plasma membrane-localized ER population is to generate rapid/extranuclear signal transduction pathways that culminate in the activation of the protein kinase cascade[7]. The nature of these pathways as well as the role played in cell functions differs between ERα and ERβ[6]. In particular, rapid signals generated from the E2-ERα complex drive cells into the cell cycle and represent the main determinant for the E2 proliferative/survival effects[8,9]. By contrast, rapid effects generated by the E2-ERβ complex drive cells out of the cell cycle[10,11], representing the key to understanding the E2-induced antiproliferative effects working both during differentiative processes and in colon adenocarcinoma[11-17].

ERs are relatively promiscuous nuclear receptors with the ability to recognize, besides E2, different exogenous substances[18]. Several of these substances such as bisphenol A (BPA), diethyl hexyl phthalate, and the plant-derived polyphenols show estrogenic activity, thus they are collectively called xenoestrogens. Besides other impairment of E2 actions, the possible contribution of xenoestrogens in the incidence of E2-related cancers has only fairly recently received attention. In particular, only a few studies addressed the putative association between increased risk of colon cancer and environmental and occupational exposures to xenoestrogens have been reported[19].

In this review, we will report recent data, including ours, on E2 action in colon health and disease discussing on how xenoestrogens could impact on these E2 effects in colon cancer. In particular, two plant-derived flavonoids (*i.e.*, naringenin, Nar, and quercetin, Que) and one synthetic food-contaminant bisphenol A (BPA) will be reported as prototype molecules to evaluate how xenoestrogens can impact on cell proliferation/apoptosis balance, the critical physiological function of E2 in colon.

EFFECT OF 17β-ESTRADIOL IN COLON

Although the colon might not be considered one of "conventional" E2 target tissue, this pleiotropic hormone exerts various actions on the organs which assemble gastrointestinal apparatus. Whereas the role of E2 in colon malignancies is well established[20] (see below), less information are available on physiological functions of E2 in the colon[21]. The impact of E2 on colon physiology became evident when considering that several gastrointestinal disorders show considerable gender-specific incidence. As an example, the predominance of constipation in women is frequently reported with a female/male ratio approximately of 9:1. Also, the prevalence of irritable bowel syndrome is higher in women compared to men suggesting the involvement of E2 in the regulation of colon motility. This evidence is also supported by studies reporting delayed gastrointestinal transit time during pregnancy, characterized by high E2 and progesterone levels[22]. Both ERα and ERβ are present in enteric nerve cells[23] and in colonic smooth muscle cells[24] sustaining the E2 potential function as intestinal motility regulator. In addition, E2 also exerts profound actions on epithelial cells of intestinal wall. An E2-dependent up-regulation of sodium/hydrogen exchanger-3 in the plasma membrane of epithelial cells of the proximal colon has been reported in pregnant mice[21,25].

Knockout experiments targeting ER genes in mice have been useful in understanding the role played by ERα and ERβ in colon. Indeed, targeted disruption of ERβ in mice[26] and further investigation of tissue expression, have revealed that ERβ is the predominant ER expressed in colonic tissues[27-29] and that its expression is selectively lost in human malignant colon tissue[6,30-32].

To better understand the physiological role of ERβ in colonic tissue, Wada-Hiraike *et al*[33] compared morphology, proliferation, and differentiation of colonic epithelium in ERβ$^{-/-}$ mice and wild-type (wt) littermates. BrdUrd labeling revealed that the number of proliferating cells was higher in ERβ$^{-/-}$ mice and that the migration of labeled cells toward the luminal surface was faster in ERβ$^{-/-}$ mice than in wt littermates. Additionally, in the absence of ERβ, there was a decrease in apoptosis and in the expression of the differentiation markers. Finally, ERβ$^{-/-}$ mice display disrupted tight junction formation and abnormal colonic architecture[33]. As a whole, the loss of ERβ leads to hyperproliferation, loss of differentiation, and decreased apoptosis in the epithelium of colon suggesting a pivotal role for ERβ in the organization and architectural maintenance of the colon[32].

EFFECT OF 17β-ESTRADIOL IN COLORECTAL CANCER

Colorectal cancer is thought to develop as a sequence from aberrant crypt proliferation or benign hyperplasia to benign adenoma and then in most cases to adenocarcinoma. Epidemiological studies ascertained that this cancer is the second to fourth most common fatal malig-

nancy in industrialized countries[33-37]. Although colorectal cancer is a common malignancy in both sexes[38], several sex-related differences in incidence, certain molecular characteristics and response to chemotherapy have been reported. In particular, the difference between sexes are more striking amongst premenopausal women and age-matched men[29,38,39]. Based on a meta-analysis of 18 epidemiologic studies, the use of hormone replacement therapy by postmenopausal women was associated with a 20% decrease in colon cancer risk[40,41]. Other studies also demonstrated that women with a history of current or past hormone replacement therapy had a significantly decreased risk of colorectal cancer and showed that there are gender differences regarding cancer location and type within the colon[4,42].

These findings suggested that exposure to E2 and/or estrogenic compounds may underlie the differences between sexes leading many investigators to search for the ER subtype involved in this form of protection exerted by E2 against colorectal cancer. Since ERα is reported to be minimally expressed in normal colon mucosa and colon cancer cells[27,43], the effects of estrogen on colon cancer susceptibility could be mediated by ERβ[13]. ERβ1, 2 and 5 have been demonstrated in normal colorectal mucosa and at much higher levels than ERα[27,30]. Using semi-quantitative reverse transcription-polymerase chain reaction, Campbell-Thompson *et al*[27] showed that ERβ is the predominant ER subtype in the human colon, and that decreased ERβ1 (ERβwt) and ERβ2 (ERβcx) mRNA levels are associated with colonic tumorigenesis in women. In accordance, other authors[28,30] showed that ERβ expression was significantly lower in colon cancer cells than in normal colonic epithelium, and that there was a progressive decline in ERβ expression, which paralleled the loss of malignant colon cell dedifferentiation. A model of mice bearing germline mutations in murine Adenomatosis polyposis coli (APC) develops multiple intestinal tumors. In this model, E2-induced prevention of APC associated tumor formation was correlated with an increase in ERβ protein and a decrease in ERα expression[13,44].

Beside the previous models, also human colon cancer cell lines have been found to express primarily ERβ[45,46], where E2 stimulation (10-1000 nmol/L) consistently induced apoptosis in a dose-dependent manner[16,17,41,46]. Altogether, these results strongly suggest that the presence of ERβ could justify the E2 effects against colon carcinogenesis.

17β-estradiol action mechanism in colorectal cancer

The first mechanism in anti-proliferative action of ERβ was suggested by the papers of Paruthiyil *et al*[14] and Ström *et al*[15]. They showed that introducing ERβ into breast cancer cell line (MCF-7 and T47D), which also expresses ERα, caused an inhibition of proliferation *in vitro* and prevented tumor formation in a mouse xenograft model in response to E2. ERβ inhibited proliferation by repressing components of the cell cycle which are associated with proliferation, such as c-myc, cyclin D1, and cyclin A gene transcription, and by increasing the expression of Cdk inhibitor $p21^{Cip1}$ and $p27^{Kip1}$, which leads to a G_2 cell cycle arrest. These findings suggested a possible role for ERβ as tumor suppressor in breast cancer, impairing ERα-mediated proliferative effects of E2[14,15]. But in colon mucosa and colon cancer cells only ERβ is expressed[27,43], so the protective effects of estrogen on this tissue should be mediated by specific ERβ-activated signal transduction pathways.

To test this hypothesis, we used DLD-1 colon adenocarcinoma cancer cells in which only ERβ1 isoform is present. In these cells ERβ undergoes palmitoyl acyl transferase-dependent S-palmitoylation which allows to a small ERβ pool to localize at the plasma membrane and associate to caveolin-1 and the p38 member of mitogen activated protein kinase (MAPK) family[16]. Upon E2 stimulation, ERβ undergoes de-palmitoylation paralleled by an increased association of receptor to caveolin-1 and to p38. The physical association ERβ-caveolin-1 and p38 increase ERβ level at the plasma membrane, impairing its association to other signaling proteins which characterize E2-induced ERα-mediated cell survival and proliferation [*i.e.,* Src, extracellular regulated kinase/mitogen activated protein kinase (ERK/MAPK), and phosphatidyl inositol 3 kinase/serine-threonine protein kinase Akt (PI3K/AKT)][16]. On the other hand, the E2-induced ERβ association to p38 strongly impacts on DLD-1 colon cancer cells growth. In fact, p38 activation is required for the activation of downstream pro-apoptotic cascade involving the cleavage of caspase-3 and of its main substrate the poly-(ADP-ribose) polymerase (PARP). Further study of DLD-1 cells, revealed that ERβ activation of the p38-MAPK pathway leads to increased expression of ERβ itself by both genomic and nongenomic means[17] leading to a self-perpetuating cycle increasing its protective effect.

As a whole, the membrane-starting signal due to the presence of ERβ at the plasma membrane seems to be mainly involved in protective effects of E2 against colorectal cancer. In fact, the treatment of these cells with the palmitoylation inhibitor 2-Bromopalmitate (2Br) completely remove ERβ from the plasma membrane impairing p38 activation. This condition prevents the pro-apoptotic cascade activation without interfering with ERβ transcriptional activity which, indeed, is still able to promote ERE-dependent gene transcription[17].

Furthermore, experimental studies with nitric oxide (NO) support the E2 rapid signal involvement in protective effects of E2 mediated by ERβ against colon cancer. NO is a diatomic molecule whose presence, modulated by several hormones including E2, is important for gastrointestinal motility. NO mainly acts through *S*-nitrosylation of cysteine (Cys) residues in target proteins modulating their activity[47-49]. Among proteins regulated by NO, modulation of ERs has been demonstrated. This molecule is able to link to ER's zinc finger impairing their transcriptional activity without interfering with rapid signal pathways. S-nitrosylation seems to selectively modulate the bioactivity of ER, shifting the receptor from its role as a transcription factor toward rapid functions. For instance, in DLD-1 colon cancer cells, in the occurrence

of NO concentration in micromolar range, normally present during peristalsis, transcriptional activity of ERβ is inhibited, but ERβ maintains its capability to mediate pro-apoptotic effects of E2 inducing caspase-3 activation and the PARP cleavage. When over produced (*e.g.*, during inflammation processes) NO worsens its effects. Although the ERβ-dependent phosphorylation of p38/MAPK is still present, NO inhibits the caspase-3 catalytic activity by nitrosylation of enzyme's Cys residues[48].

Thus, besides its role as negative modulator of ERα activities above reported and its ability to decrease the transcription of anti-apoptotic genes[50], these findings indicate that ERβ triggers specific rapid signal cascade mainly involved in protective effect of E2 in colorectal cancer.

XENOESTROGEN EFFECT IN COLORECTAL CANCER

Xenoestrogens, like other endocrine disrupting substances, could interfere with the synthesis, secretion, transport, metabolism, binding, action or elimination of E2[51,52]. All these actions could affect the homeostasis maintenance, reproduction, and developmental processes regulated by this hormone in all organs and tissues including colon. Currently quite lot chemicals, containing halogen groups have been identified as xenoestrogens. They include: (1) synthetic chemicals used in industry, agriculture, and consumer products; (2) synthetic chemicals used as prescription drugs; and (3) chemical components of human and animal food. Xenoestrogens have very low water solubility, extremely high lipid solubility, and long environmental half-life resulting in a continue increase of their global concentration in the environment even at great distances from where they are produced, used or released. Exposure to xenoestrogens can occur from a number of different sources: water, air, food, soil or even in the workplace[53].

In a review embracing environmental and occupational causes of cancer, Clapp *et al*[19] identified only a few studies that found increased risk of colon cancer associated with environmental and occupational exposures. The researchers reported a study in a nested case-control study of female textile workers in Shanghai showing that long-term exposure (20 years or longer) to dye and dye metabolites resulted in nearly 4-fold elevation in colon cancer risk. In a cohort of aerospace workers exposed to hydrazine, a component of rocket fuels, colon cancer was elevated when exposures were lagged 20 years and risk significantly increased with increasing dose. Lastly, a significant increase in colon cancer risk was reported among pesticide applicators with increasing level of exposure to the herbicide dicamba. Although these limited studies indicate a positive correlation between colon cancer incidence and environmental pollutants, no information on the estrogenicity of these compounds was reported[19].

In a recent and very interesting review, Sokolosky and Wargovich[54] reported and commented the data by GLOBOCAN[36]. The researchers evidenced that the incidences for colorectal cancer, as well as most other cancers, were highest in Australia, Canada, Western Europe, Japan, and the United States, while the lowest incidences were reflected for the majority of the African continent (except for South Africa), India, the Middle East, and South American countries surrounding the Amazon basin[54]. Although the reduced risks of cancer and other chronic diseases reported in people from these low-income countries could be attributable in some ways to genetic disposition, it could be also related with environmental factors arising from their retention of preventive dietary and lifestyle practices. Thus, they concluded that the correlation between modernization, acculturation, and increased risk for chronic diseases such as colorectal cancer exists[54].

Recently, two Scientific Statement of The Endocrine Society focused on a demanding need to understand the basic mechanisms of action and the physiological consequences of endocrine disruptors. In particular, among other scientific recommendations for research, it is imperative to perform basic *in vitro* molecular studies to identify pathways for xenoestrogens influence on endocrine tissues[51,52]. Given the diverse repertoire of xenoestrogens present in the environment, it should not be surprising that these molecules exert their effects through several mechanisms. Indeed, xenoestrogens act directly *via* steroid hormone receptors or indirectly through non-steroid receptors (*e.g.*, neurotransmitter receptors such as the serotonin receptor, dopamine receptor, norepinephrine receptor), orphan receptors (*e.g.*, aryl hydrocarbon receptor AhR), and on enzymatic pathways involved in steroid biosynthesis and/or metabolism[53]. Therefore, such considerable structure heterogeneity and diverse potential mechanism of action make the characterization of the effects of these substances quite hard. Nonetheless, many xenoestrogens often have a phenolic moiety that mimics E2 enabling them to interact with ERs as agonists or antagonists[18]. However, xenoestrogens have been often, if not exclusively, tested for their ability to influence the ERs nuclear activities while xenoestrogens ability to participate in the extranuclear activities of the ERs has been rarely evaluated. It has been reported that BPA, a well known xenoestrogen, binds to ERα and ERβ with a lower affinity than E2 (*i.e.*, 10 μmol BPA *vs* 10 nmol E2) inducing E2-responsive gene expression. Interestingly, the set of genes induced by BPA and E2 seems to be quite different, most of them being unique for BPA[55-57]. Moreover, our recent experiments in colon cancer cells expressing only ERβ subtype indicates that BPA acts as a full E2 antagonist by blocking both genomic and extranuclear ERβ activities which drive colon cancer cells to apoptosis[57].

The plant-derived flavonoids represent a singular class of xenoestrogens. Indeed, over several decades, a combination of epidemiological and experimental indications has shown that these compounds have a protective potential on human health[58-61]. This evidence led to a substantial increase in flavonoid usage as dietary

components, even if the estrogen-like or the estrogen antagonistic effects are not yet fully clarified. Intriguingly, flavonoids are considered potentially able to exert also a protective role against the development of E2-dependent tumours by binding to ERα and ERβ[59,62-65]. Among others, nutritionally relevant concentrations of naringenin (5,7,4'-trihydroxyflavone, Nar), especially abundant in oranges and tomatoes, or of quercetin (2-(3,4-dihydroxyphenyl-3,5,7-trihydroxy-4*H*-1-benzopyran-4-one, Que) present in apples, onions, and other vegetables induce apoptosis in different cancer cell lines containing ERα or ERβ (*e.g.*, colon, breast, and uterus cancer cell lines)[62-64]. As an example, quercetin, at nutritionally relevant concentrations, mimic E2-induced apoptotic effect in ERβ-containing DLD-1 colon cancer cell lines by inducing the activation of p38/MAPK. In turn, p38 activation is responsible for the pro-apoptotic activation of caspase-3 and the cleavage of PARP. Notably, no inactivation or downregulation of the survival kinases (*i.e.*, PI3K/AKT and ERK/MAPK) or the antiapoptotic protein Bcl-2 was observed after quercetin stimulation[64]. On the contrary, quercetin acted similarly to E2 by increasing the levels of the oncosuppressor protein PTEN and by impeding ERβ-dependent cyclin D1 promoter activity, which subsequently resulted in the transcription of the estrogen-responsive element remaining unchanged[64]. As a whole, these data indicate that flavonoids mimic the E2 effects in the presence of ERβ1, thus maintaining the E2 anticarcinogenic potential against colorectal cancer. Intriguingly, even in the presence of BPA naringenin impairs cancer cell proliferation by activating caspase-3-dependent apoptosis, at least in E2-dependent breast cancer cell lines expressing ERα[66]. If similar mechanisms are working also in colorectal cancer cell lines is unknown at the present.

CONCLUSION

The increase in non-communicable diseases in humans and wildlife over the past 40 years indicates an important role of the modernization and its resulting life style trends in disease etiology. Over the years this concern grew with the advancement of biochemical, biomedical, and biotechnological industries and with the increasing possibility of bioterrorism and chemical-warfare. The man-made chemicals and, in particular xenoestrogens, are nowadays found abundantly in the environment on residential buildings, cars, furniture, plastics, products such as baby feeding bottles, lining, tin-food containers, and even in children's toys. Thus, xenoestrogens are important component of the environmental influences on disease, along with nutrition and other factors. This sentence is sustained by data obtained from epidemiologic evidence, *in vivo*, and *in vitro* studies which give us an alarming picture of the wide effect of xenoestrogens on human health[47,48,62]. In particular, the literature demonstrates a role of these substances in the pathogenesis of obesity, diabetes mellitus, cardiovascular disease, and cancer the major epidemics of the modern world[67-69].

Here, we explored the idea that the increased incidence of diseases such as colorectal cancer could be the result of physiological and molecular imbalances of E2 signals. Flavonoid-deprived diets combined with low-dose exposures to xenoestrogens could be linked to increasing incidences of this type of cancer in Westernized societies and developing countries. In order to address a disease multi-factorial, case-specific, and remarkably adaptive as colorectal cancer, research must focus on its root causes in order to elucidate the molecular mechanisms by which they can be prevented or counteracted *via* plant-derived compounds such as naringenin and quercetin. As a whole, the research on the impact of xenoestrogens on E2-induced protection against colorectal cancer represents an area that requires further investigation.

At the present, a huge amount of literature assembles tissue culture, animal studies (*in vivo* and *ex vivo*), and epidemiological data only on the effect of xenoestrogens on gynaecological cancers (*i.e.*, breast, ovary, and endometrial cancers) whereas only few address the role of these compounds on colorectal cancer. In addition, data on xenoestrogen action mechanisms in colorectal cancer are still unclear and confused. Molecular studies *in vitro* and with *in vivo* animal models are needed to identify pathways for xenoestrogen influence on this E2 target tissue. In addition, studies on xenoestrogens on gastrointestinal and colon are much underrepresented, and these fields need to be expanded.

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ORIGINAL ARTICLE

Autophagy inhibition by chloroquine sensitizes HT-29 colorectal cancer cells to concurrent chemoradiation

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Abstract

AIM: To investigate whether the inhibition of autophagy by chloroquine (CQ) sensitizes rectal tumors to radiation therapy (RT) or concurrent chemoradiation (chemoRT).

METHODS: *In vitro*, HCT-116 and HT-29 colorectal cancer (CRC) cell lines were treated as following: (1) PBS; (2) CQ; (3) 5-fluorouracil (5-FU); (4) RT; (5) CQ and RT; (6) 5-FU and RT; (7) CQ and 5-FU; and (8) 5-FU and CQ and RT. Each group was then exposed to various doses of radiation (0-8 Gy) depending on the experiment. Cell viability and proliferative capacity were measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and clonogenic assays. Clonogenic survival curves were constructed and compared across treatment groups. Autophagy status was determined by assessing the LC3-Ⅱ to LC3-Ⅰ ratio on western blot analysis, autophagosome formation on electron microscopy and identification of a perinuclear punctate pattern with GFP-labeled LC3 on fluorescence microscopy. Cell cycle arrest and cell death were evaluated by FACS and Annexin Ⅴ analysis. All experiments were performed in triplicate and statistical analysis was performed by the student's *t* test to compare means between treatment groups.

RESULTS: RT (2-8 Gy) induced autophagy in HCT-116 and HT-29 CRC cell lines at 4 and 6 h post-radiation, respectively, as measured by increasing LC3-Ⅱ to LC3-Ⅰ ratio on western blot. Additionally, electron microscopy demonstrated autophagy induction in HT-29 cells 24 h following irradiation at a dose of 8 Gy. Drug treatment with 5-FU (25 μmol/L) induced autophagy and the combination of 5-FU and RT demonstrated synergism in autophagy induction. CQ (10 μmol/L) alone and in combination with RT effectively inhibited autophagy and sensitized both HCT-116 and HT-29 cells to treatment with radiation (8 Gy; $P < 0.001$ and 0.00001, respectively). Significant decrease in clonogenic survival was seen only in the HT-29 cell line, when CQ was combined with RT at doses of 2 and 8 Gy ($P < 0.5$ and $P = 0.05$, respectively). There were no differences in cell cycle progression or Annexin V staining upon CQ addition to RT.

CONCLUSION: Autophagy inhibition by CQ increases CRC cell sensitivity to concurrent treatment with 5-FU and RT *in vitro*, suggesting that addition of CQ to chemoRT improves CRC treatment response.

Key words: Autophagy; Chloroquine; Radiosensitization; Colorectal cancer

Core tip: Autophagy is implicated as a mechanism of resistance to cancer treatment. We hypothesized that chloroquine, a lysosomotropic autophagy inhibitor, would sensitize colorectal cancer (CRC) cell lines to both radiation therapy (RT) alone and concurrent chemoradiation. Our results showed that chloroquine decreased clonogenic survival of CRC cells when given in combination with RT or concurrent 5-fluorouracil and RT. Radiosensitization by chloroquine represents a novel therapeutic approach to enhance treatment efficacy in rectal cancer.

Schonewolf CA, Mehta M, Schiff D, Wu H, Haffty BG, Karantza V, Jabbour SK. Autophagy inhibition by chloroquine sensitizes HT-29 colorectal cancer cells to concurrent chemoradiation. *World J Gastrointest Oncol* 2014; 6(3): 74-82 Available from: URL: http://www.wjgnet.com/1948-5204/full/v6/i3/74.htm DOI: http://dx.doi.org/10.4251/wjgo.v6.i3.74

INTRODUCTION

In 2013 it is estimated that 40340 new cases of rectal cancer will be diagnosed in the United States[1]. The standard of care for patients with locally advanced rectal cancer consists of pre-operative 5-fluorouracil (5-FU) and radiation therapy (RT), followed by surgical resection. Five-year survival rates vary drastically depending on pathologic response after neoadjuvant treatment, from 85%-90% in patients with a pathologic complete response (pCR) to 66% in patients without pCR[2,3]. Therefore, improvements in the efficacy of pre-operative treatment for locally advanced rectal cancer have great potential to significantly impact patient survival.

Autophagy, a lysosomal degradation process of cellular organelle and protein recycling under stressful conditions, has been implicated as a cancer cell survival mechanism. Increased levels of autophagy have been observed in nutrient- and oxygen-poor tumor regions as compared to highly vascularized, nutrient-enriched areas[4]. Autophagy induction in metabolically stressed tumor regions allows cancer cells to generate new substrates for growth through recycling of "self" material. Autophagy also supports the increased metabolic needs of Ras-mutant cancer cells by providing substrates for oxidative phosphorylation[5] and several studies have suggested that the tumorigenic potential of Ras-transformed tumor cells is highly dependent on autophagy[5]. Autophagy induction in hypoxic tumor cores has been proposed as a mechanism of resistance to chemotherapy and radiation. During RT, intermittent hypoxia occurs in association with a significant increase in the level of reactive oxygen species and concomitant stabilization of HIF-1α under aerobic conditions[6-8]. By targeting the compensatory and prosurvival mechanism induced in response to tumor hypoxia in RT-treated neoplasms, autophagy inhibition may improve the efficacy of treatment.

Given that autophagy is a mechanism of resistance to both chemotherapy and radiotherapy, the addition of chloroquine (CQ), an inhibitor of autophagy, may allow for improvements in tumor responsiveness. An earlier study demonstrated the anti-cancer effect of CQ with 5-FU in CRC cells[9]. As expected, 5-FU inhibited CRC cell proliferation through cell cycle arrest and, to a lesser degree, apoptosis. This effect was potentiated by CQ and autophagy induction was demonstrated by increased acidic vesicles and increased LC3-Ⅱ expression. While CQ demonstrated synergism with chemotherapy, its actions with respect to RT in CRC require exploration. Understanding autophagy's role in CRC radioresistance is critical and has the potential to create new opportunities for therapeutic intervention.

The purpose of this study is to provide data and rationale for the application of autophagy inhibition in the treatment of localized rectal cancer by adding hydroxychloroquine to routine 5-FU and RT. We hypothesized that autophagy inhibition by CQ with standard chemoRT for rectal cancer will enhance radiosensitization. We tested our hypothesis by characterizing the effects of radiation on autophagy in CRC cells and evaluating the efficacy of combination treatment with CQ, 5-FU and radiation.

MATERIALS AND METHODS

Cell lines and culture

HCT-116 and HT-29 CRC cell lines (ATCC) were maintained in McCoy's 5A (GIBCO, Invitrogen, New York, United States) medium containing 10% fetal bovine serum (GIBCO) and 1% penicillin/streptomycin (GIBCO). Cells were incubated at 37 ℃ with 5% CO_2.

Drug and radiation treatment

Drug treatments included the following groups: (1) Control group, vehicle; (2) CQ (Sigma Aldrich); (3) 5-FU (Sigma Aldrich); (4) RT (Gammacell 40 Exactor, Best Theratronics); (5) CQ and RT; (6) 5-FU and RT; (7) CQ and 5-FU; and (8) 5-FU and CQ and RT.

Electron microscopy

Cells were harvested by trypsinization, fixed in 2.5% gluteraldehyde/4% paraformaldehyde in 0.1 mol/L cacodylate buffer, then post-fixed in 1% osmium tetroxide buffer. After acetone dehydration, cells were embedded in spur resin. Thin sections (90 nm) were cut on a Reichert Ultracut E microtome and stained with saturated uranyl acetate and lead citrate solution. Sections were examined at 80 kV with a JEOL 1200EX transmission electron microscope (TEM).

Fluorescence microscopy

GFP-labeled LC3 plasmid and a GFP-expressing control plasmid were transiently transfected into HCT-116 and HT-29 cell lines using Lipofectamine 200 (Invitrogen). Cells attached overnight, and were treated with Rapamycin (200 nmol/L), or RT (2-8 Gy) ± 5-FU (15 μmol/L for HT-29 cells, 25 μmol/L for HCT-116 cells). Six hours

later, cells were washed with PBS and fixed with 10% Formalde-Fresh Solution (Fisher Scientific). Cells were mounted with Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA) and GFP fluorescence was examined at 60 × magnification. Autophagy was quantified by percentage of cells exhibiting a perinuclear punctate pattern per 50 cells.

Cell viability assays

Cells were plated into 96-well plates (2.5×10^3 cells/well). After overnight incubation, cells were incubated with either media without drug or media containing CQ (10 μmol/L), 5-FU (25 μmol/L) or both. Each group of drug-treated cells was irradiated (0-8 Gy) within 1 h of drug exposure. Following irradiation, cells were incubated for 72 h and cell viability was measured using 50 μL of 0.5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution. Medium was removed after 4 h of incubation at 37 ℃ and 250 μL of DMSO was used to dissolve the blue MTT formazan precipitate. Absorbance was measured at 560 nm on a Victor plate reader. Cell survival was calculated relative to untreated cells.

Colony forming assays

HT-29 cells were plated at concentrations of 150, 350, 600, and 20×10^3 cells per 10-cm plate for irradiation at 0, 2, 4 and 8 Gy, respectively. HCT-116 cells were plated at concentrations of 400, 2.5×10^3, 5×10^3 and 250×10^3 cells per 10-cm plate for irradiation at 0, 2, 4 and 8 Gy, respectively. After plating and overnight incubation, cells were treated with drug combinations followed by radiation within 1 h. Then HT-29 and HCT-116 cells were incubated for 14 and 7 d, respectively. Cells were washed with PBS and stained with 50% methylene blue for 30 min. Colonies were counted positive if they contained > 50 cells.

Western blot analysis

After plating and overnight incubation, cells were treated with CQ, 5-FU or both, and then irradiated within 1 h at 2-8 Gy. At time points of 30 min, 1 h, 2 h, 4 h, 6 h and 24 h after irradiation, cells were harvested by scraping technique and stored as a pellet at -80 ℃. Cell lysis buffer with protease inhibitor cocktail was added to each sample, followed by sonication. Protein concentrations were quantified using Bio-Rad Protein Assay. Equal amounts of protein (12.5 μg) were loaded onto 4%-20% Tris-Glycine PAGE gels (Invitrogen, New York, United States) and run using the Invitrogen XCell SureLock system. Proteins were transferred onto PVDF paper and blots were blocked with 0.25% Milk in TBST using Milipore SnapID. Primary antibodies to LC3 (rabbit, Novus Biologicals, dilution 1:10000) and p62 (mouse, MBL, dilution 1:10000) were incubated overnight at 4 ℃. Blots were washed with 1X TBS with 0.1% Tween using Millipore SnapID and then incubated at room temperature for 1 h with secondary antibody, goat anti-rabbit (CalBio, 1:10000) or goat anti-mouse (CalBio, dilution 1:10000) in 0.25% milk in TBST. Pierce ECL Western Blotting Substrate was used to visualize proteins and expression was quantified using ImageJ software.

Cell cycle analysis

Cells were treated according to the treatment groups previously described. Within 1 h of drug treatment, cells were irradiated at 8 Gy. After incubation for 24 and 48 h, floating and adherent cells were collected, washed with PBS, fixed with ice-cold 70% ethanol and stained with propidium iodide (PI, 50 μg/mL). Cell cycle progression was analyzed using a Cytomics FC500 Analyzer (Beckman Coulter, Brea, Ca). Gating was used to remove cellular debris and fixation artifacts.

Apoptosis

At 48 h after drug and RT, floating cells and trypsinized adherent cells were collected. Cells were stained with PI and Annexin V, per manufacturer's instructions. Cells, which stained positive for Annexin V, but negative for PI, were considered early apoptotic.

Statistical analysis

Experimental results were reported as a mean of at least three independent experiments conducted in triplicate. Statistical analysis was performed by a two-tailed Student's *t* test and a P value < 0.05 was considered statistical significant.

RESULTS

Radiation-induced autophagy

Irradiation induced autophagy in both HCT116 and HT-29 cell lines (Figure 1). TEM demonstrated autophagy induction in HT-29 cells 24 h following irradiation at a dose of 8 Gy (Figure 1A). Compared to non-irradiated controls, RT-treated cells exhibited increased autophagosome formation, as illustrated by increased numbers of double membrane vesicles (Figure 1A, inset).

Western blotting for the autophagosome-associated protein light chain 3 (LC3) confirmed autophagy induction at multiple time points following irradiation (Figure 1). Conversion of cytosolic LC3- I to the proteolytically cleaved and phosphatidyl-ethanolamine (PE)-conjugated, membrane bound form LC3-Ⅱ occurs during autophagosome formation and increased LC3-Ⅱ: I ratio is considered a marker of autophagy induction[10]. Increased LC3-Ⅱ: I ratios were seen in HT-29 and HCT-116 cell lines at early time points, namely at 4 and 6 h post-RT, respectively (Figure 1B). RT doses of 4 and 8 Gy induced autophagy in HT-29 cells, while HCT-116 cells showed autophagy induction at 2, 4 and 8 Gy. By 24 h following RT, autophagy induction, as indicated by increased LC3-II:I ratio, was only seen in HT-29 cell lines and occurred across all radiation doses, from 2 to 8 Gy, (Figure 1C).

To further investigate the effects of radiation on autophagic response, HCT-116 and HT-29 cell lines were transiently transfected with GFP-labeled LC3 plasmid and examined for green fluorescent LC3 puncta, representing autophagosomes. Increasing RT doses significant-

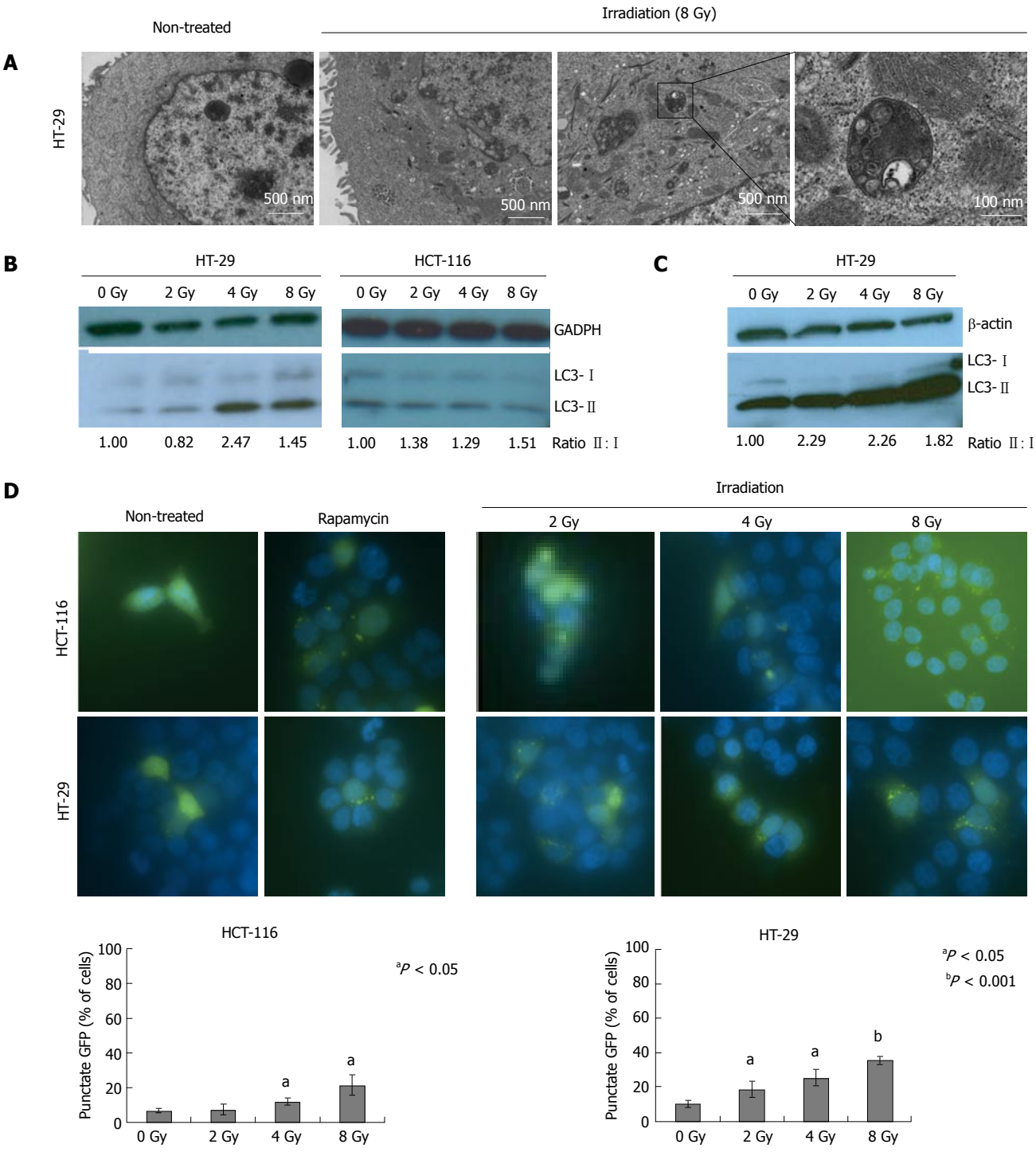

Figure 1 Radiation-induced autophagy. A: HT-29 cells were irradiated (8 Gy) and autophagy induction was assessed by the formation of double membrane autophagosomes on transmission electron microscopy 24 h post-treatment; B: Western blot analysis of LC3 expression demonstrates increased LC3-Ⅱ: Ⅰ ratio and autophagy induction at 4 and 6 h post-irradiation for HT-29 and HCT116 cells, respectively; C: Increased LC3Ⅱ: Ⅰ ratios are seen 24 h post-irradiation at 2-8 Gy for HT-29 cells; D: GFP-labeled LC3 puncta develop within 6 h of treatment with Rapamycin (200 nmol/L) or radiation (2-8 Gy) in both HCT116 and HT-29 cell lines. Bar graphs represent quantification of percentage of cells with perinuclear punctate pattern 6 h following radiation.

ly increased the number of cells with GFP punctate pattern compared to untreated controls for both HCT-116 and HT-29 cell lines ($P < 0.05$) (Figure 1D).

Chemoradiation-induced autophagy

Previous studies demonstrated autophagy induction in HCT-116 and HT-29 cells following treatment with 5-FU alone[9,11,12]. We now examined the impact of concurrent treatment with 5-FU and RT on autophagy functional status in CRC cells. ChemoRT resulted in increased autophagy induction in HT-29 cells compared to 5-FU alone (Figure 2A and B) and may have had a synergistic effect at higher RT doses (8 Gy) (Figure 2B). Autophagy induction in HT-29 cells following chemoRT was also

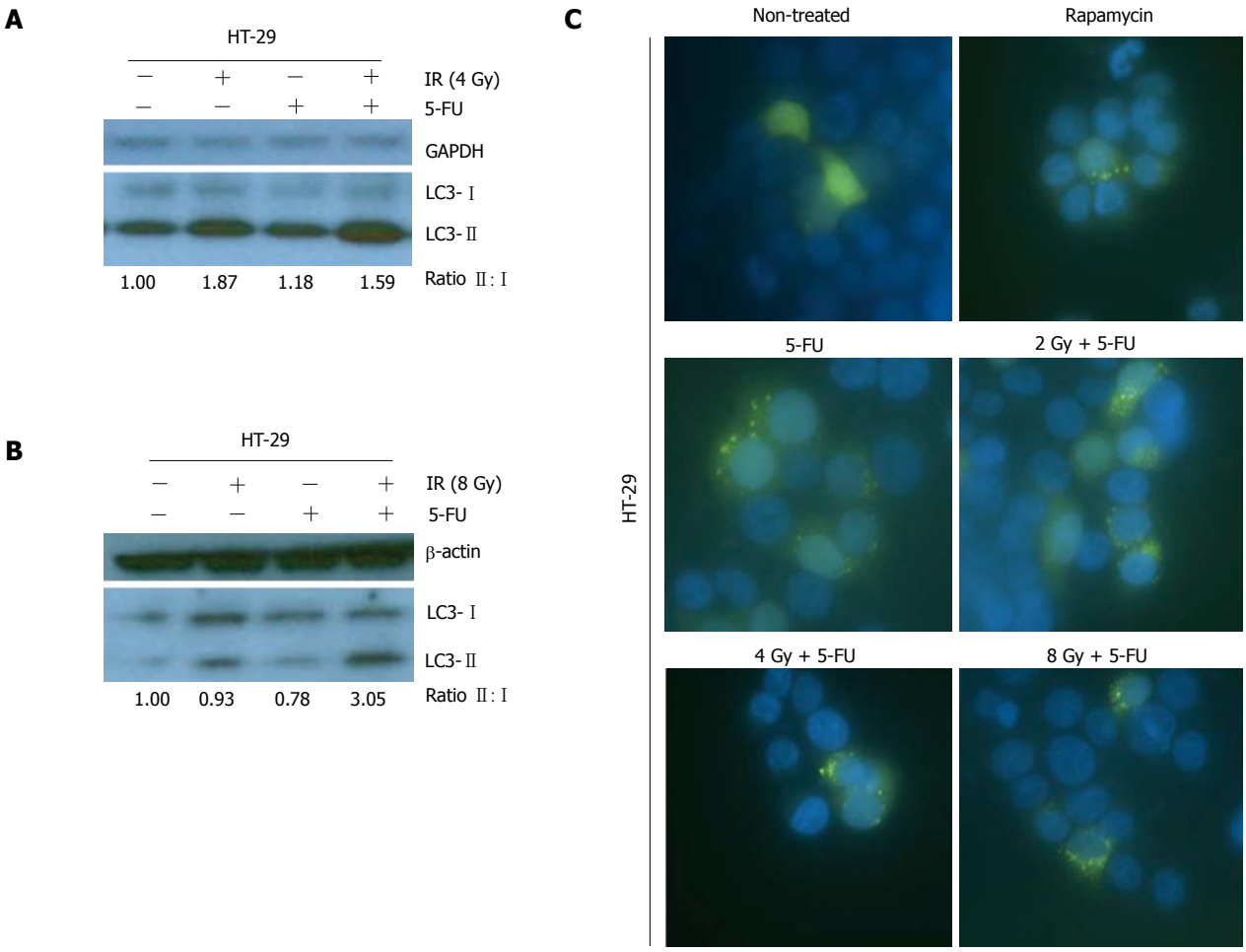

Figure 2 Chemoradiation-induced autophagy. A: Western blot of LC3 in HT-29 cells harvested 24 h after treatment with RT (4 Gy), 5-FU (15 μmol/L) or 5-FU and RT; B: Western blot of LC3 in HT-29 cells harvested 24 h after treatment with RT (8 Gy), 5-FU (15 μmol/L) or 5-FU and RT; C: GFP-labeled LC3 fluorescence in HT-29 cells fixed 6 h after treatment with Rapamycin (200 nmol/L), 5-FU (15 μmol/L) or combination RT (2-8 Gy) and 5-FU (15 μmol/L). RT: Radiation therapy; 5-FU: 5-fluorouracil.

qualitatively assessed by fluorescence microscopy (Figure 2C). GFP-fluorescent puncta formation confirmed 5-FU induced autophagy as previously reported[9,11,12] and demonstrated that chemoRT resulted in more robust autophagy upregulation.

Radiosensitization of CRC cell lines through autophagy inhibition by chloroquine

To investigate whether autophagy inhibition by CQ increases the radiosensitivity of CRC lines, we first used MTT assays (Figure 3A). Cell viability of HCT-116 cells at 72 h post-RT was significantly decreased upon addition of CQ (10 μmol/L) just prior to irradiation at 4 and 8 Gy ($P < 0.001$). Significant decreases in cell viability in the presence of CQ (10 μmol/L) in HT-29 cells were seen at 8 Gy ($P < 0.001$).

Cancer cell proliferation after treatment was examined by clonogenic survival assays. For HCT-116 cells, clonogenic survival was similar under RT alone or RT and CQ (Figure 3B), whereas HT-29 cells showed decreased survival after combination treatment with RT and CQ (0.5 μmol/L) compared to RT alone at doses of 2 and 8 Gy ($P < 0.05$ and $P = 0.05$, respectively), further supporting the MTT assay results that showed radiosensitization of CRC cell lines by CQ.

Chloroquine inhibits the last phase of autophagy by changing the pH of lysosomes, thus rendering them nonfunctional and unable to process proteins[10]. Effective autophagy inhibition by CQ is manifested as LC3-Ⅱ accumulation due to failure to re-process LC3-Ⅱ back into LC3-I[10]. As shown in Figure 3C, single agent CQ increased LC3-Ⅱ levels in HT-29 cells compared to vehicle treatment, demonstrating that CQ effectively blocked autophagic flux at the concentration used. Furthermore, HT-29 cells irradiated at 8 Gy after exposure to CQ showed increased LC3-Ⅱ accumulation compared to cells treated with CQ alone (Figure 3C), indicating that RT induced autophagy.

To investigate the mechanism underlying radiosensitization of HT-29 cells by CQ, cell death by apoptosis and cell cycle progression were assessed. CQ addition to RT (8 Gy) increased PARP cleavage, but had little effect on cleaved caspase-3 levels, compared to RT alone (Figure 3C), suggesting that concurrent use of CQ likely increased the RT-induced DNA damage response through PARP, but alternative cell death pathways other

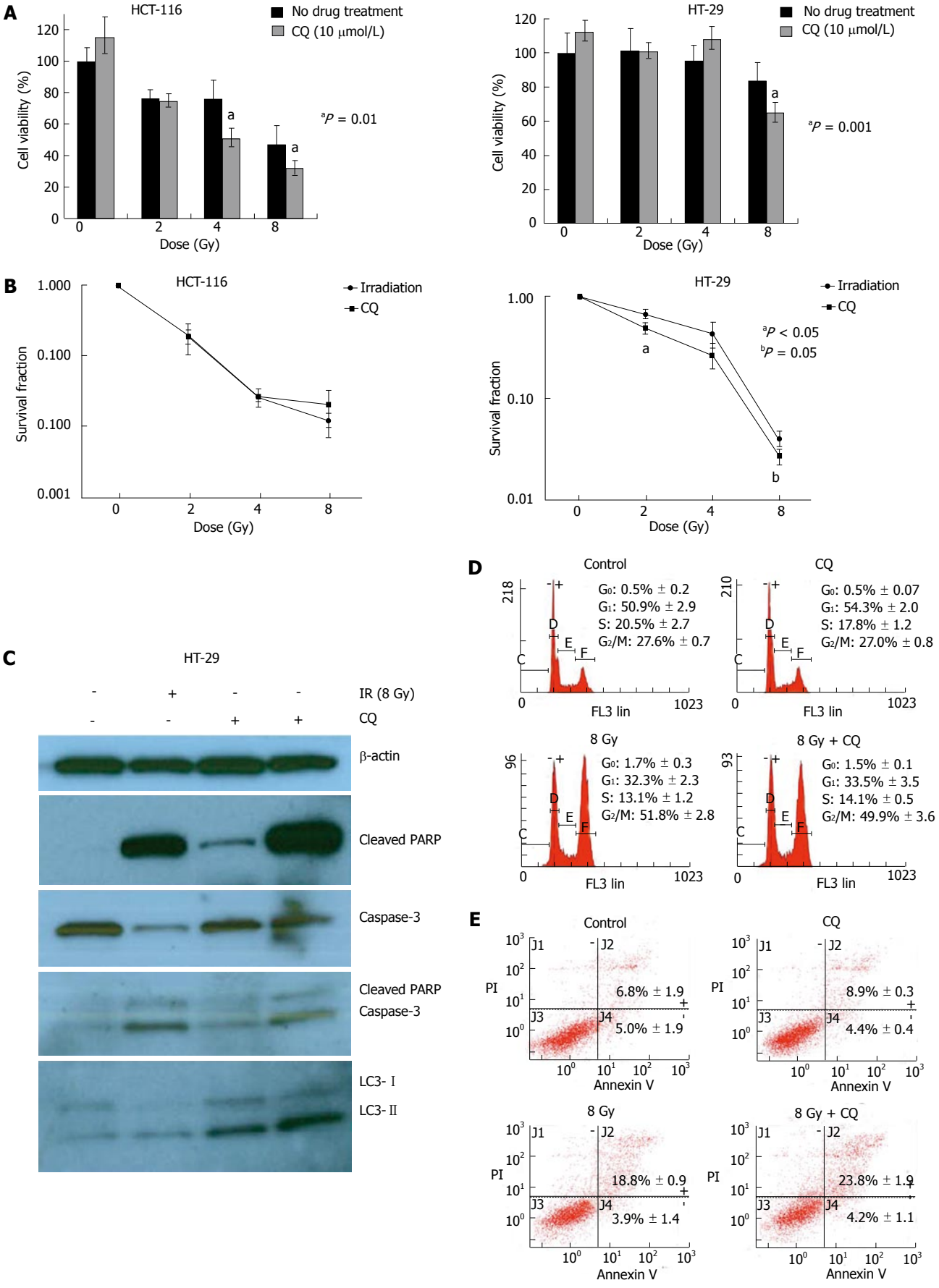

Figure 3 Radiosensitizing effects of autophagy inhibition by chloroquine. A: MTT assays in HCT-116 and HT-29 cell lines 72 h after treatment with CQ (10 μmol/L) and RT (2-8 Gy); B: Clonogenic survival of HCT-116 and HT-29 cells following treatment with CQ (0.1 and 0.5 μmol/L, respectively) and/or RT (2-8 Gy); C: Western blot in HT-29 cells harvested 24 h post-treatment with CQ (10 μmol/L) and/or RT (8 Gy); D: FACS analysis in HT-29 cells 24 h following treatment with CQ (10 μmol/L) and/or RT (8 Gy); E: Annexin V and PI staining in HT-29 cells 24 h following treatment with CQ (10 μmol/L) and/or RT (8 Gy). CQ: Chloroquine; RT: Radiation therapy.

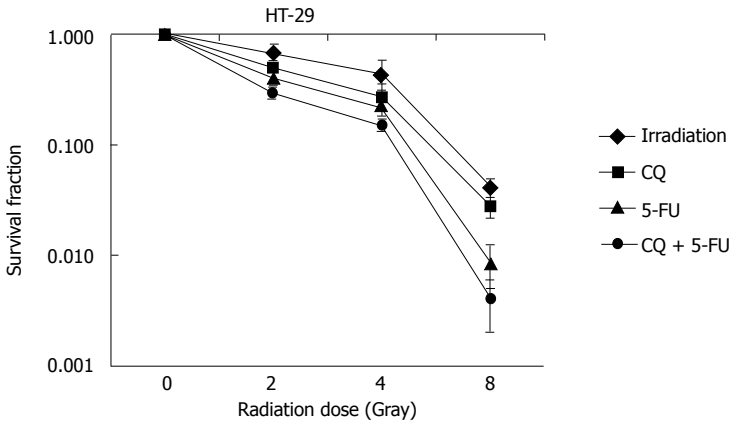

Figure 4 Chloroquine sensitizes colorectal cancer cells to chemoradiation. Clonogenic survival of HT-29 cells treated with RT (2-8 Gy) and CQ (0.5 μmol/L), 5-FU (0.5 μmol/L) or CQ and 5-FU. CQ: Chloroquine; 5-FU: 5-fluorouracil.

than apoptosis were responsible for decreased HT-29 cell survival upon radiosensitization by CQ. Cell cycle analysis demonstrated that CQ did not alter the proportion of cells in any phase of the cell cycle (Figure 3D). Flow cytometry for Annexin V and PI at 48 h after treatment (Figure 3E) confirmed that CQ did not affect early apoptosis in irradiated HT-29 cells, as the AnnexinV^+/PI cell population remained stable. Of note, addition of CQ to RT significantly increased the number of cells staining positive for both Annexin V and PI, as compared to RT alone ($P < 0.05$). Since Annexin V^+/PI^+ cells are necrotic or in late apoptosis, the radiosensitization of HT-29 cells by CQ may result from increased necrosis rather than apoptosis or cell cycle arrest.

Autophagy inhibition increases cancer cell response to chemoradiation

To investigate whether autophagy inhibition by CQ increased the therapeutic efficacy of chemoRT in rectal cancer, we examined the clonogenic survival of HT-29 cells treated with CQ (0.5 μmol/L) in combination with 5-FU (0.5 μmol/L) and/or radiation (2-8 Gy) (Figure 4). CQ addition to chemoradiation at 2 and 4 Gy significantly sensitized HT-29 cells to treatment and decreased their clonogenic survival ($P < 0.05$), whereas at 8 Gy, CQ resulted in decreased clonogenic survival showing a trend toward statistical significance compared to chemoRT alone ($P = 0.12$).

DISCUSSION

Autophagy is an evolutionarily conserved, self-digestive process in which proteins and other cytoplasmic material are recycled to support cell survival under stressful conditions (*i.e.*, cancer therapy)[10]. Autophagy has been proposed as a mechanism of resistance to RT and chemotherapy[13]. Autophagy inhibition using siRNAs against AuTophaGy (ATG)-related genes sensitizes human breast, pharyngeal, cervical, lung, and rectal carcinoma cells to RT[14]. Chloroquine, as an indirect autophagy inhibitor, renders CRC cells more sensitive to 5-FU[9], but its effects on chemoRT had not been previously explored. We hypothesized that autophagy inhibition by CQ may deprive CRC cells of an essential survival mechanism and radiosensitize treatment-resistant regions of rectal tumors.

We examined autophagic flux in HT-29 and HCT-116 cells following RT. Autophagy induction occurred in both cell lines post-radiation at early time points (Figure 1B), but was sustained at 24 h only in HT-29 cells (Figure 1C). Earlier reports of 5-FU-induced autophagy were confirmed in our study, and treatment of HT-29 cells with the combination of 5-FU and RT upregulated autophagy more than either treatment alone (Figure 2B). These results demonstrate that 5-FU and radiation, both individually and potentially synergistically when in combination, induce the prosurvival autophagic pathway in CRC cell lines, particularly HT-29 cells.

We also examined whether autophagy inhibition by CQ sensitized CRC cells to 5-FU alone, radiation alone and combined chemoRT. Increased LC3-Ⅱ values confirmed that CQ effectively inhibited RT-induced autophagy in HT-29 cells (Figure 3C), and, thus, the radiosensitization of these cells by CQ can be attributed to the CQ-mediated autophagy inhibition. Furthermore, inhibition of autophagy by CQ sensitized HT-29 cells to concurrent chemoradiotherapy (Figure 4), thus indicating that addition of CQ to chemoRT enhances treatment efficacy in CRC.

In our study, we also examined cell death mechanisms possibly underlying the decreased survival of HT-29 cells, following treatment with CQ and radiation. Although significant differences in cell cycle progression or apoptosis were not observed (Figure 3D), FACS analysis indicated increased necrosis upon addition of CQ to RT. Further studies will be needed to determine whether programmed necrosis (necroptosis) plays a role in the decreased survival of CQ- and RT-treated HT-29 cells.

Our results showed radiosensitization by CQ in HT-29 (p53-mutant) cells, but not HCT-116 (p53-wild type) cells. While these cell lines cannot be directly compared solely on the basis of their p53 status, the differences in sensitization is an observation needing further investigation. Autophagy is regulated by several signal transduction pathways, including mTOR[10] and p53[15]. In particular, p53 inhibits autophagy through various mechanisms in CRC cells[16,17], and its ablation in HCT-116 cells results in autophagy induction and resistance to irinote-

can[18]. Further investigation into the relationship between p53 and the autophagic pathway following RT needs to be conducted, as p53 status may play a role in susceptibility to radiosensitization by CQ.

In conclusion, autophagy inhibition by CQ enhanced the radiosensitivity of CRC cells and improved the therapeutic efficacy of chemoRT in CRC *in vitro*, strongly suggesting that adding hydroxychloroquine to the pre-operative regimen of 5-FU and radiation in locally advanced rectal cancer may improve treatment response. Further studies examining the anti-tumor effects of CQ-mediated autophagy inhibition should be performed in xenograft tumor models to elucidate the impact of autophagy on chemoRT responsiveness *in vivo*. Additional studies are needed to elucidate the molecular mechanisms responsible for the radiosensitizing effects of autophagy inhibition, as these results could form the basis for rationally selecting patients who may benefit most from pharmacologic autophagy modulation.

COMMENTS

Background

Standard of care for patients diagnosed with locally advanced rectal tumors consists of a pre-operative regimen of 5-fluorouracil (FU) and radiation therapy (RT). Five-year survival rates vary drastically depending on pathologic response after neoadjuvant chemoradiation; 66% survival in patients without a pathologic complete response and 85%-90% in patients with a pathologic complete response. The aim of this study was to evaluate whether the addition of an autophagy inhibitor such as chloroquine to the preoperative regimen of chemoradiation (chemoRT) could improve the efficacy of treatment.

Research frontiers

Autophagy has been proposed as a mechanism of resistance to RT and chemotherapy and autophagy inhibition using siRNAs against AuTophaGy (ATG)-related genes sensitizes human breast, pharyngeal, cervical, lung, and rectal carcinoma cells to radiation therapy. Chloroquine, as an indirect autophagy inhibitor, renders colorectal cancer cells more sensitive to 5-FU, but its effects on radiation and chemoRT had not been previously explored.

Innovations and breakthroughs

Autophagy inhibition by chloroquine increases HT-29 colorectal cancer cell sensitivity to concurrent treatment with 5-FU and RT *in vitro*.

Applications

The results of this study provide data and rationale for the clinical application of autophagy inhibition in the treatment of locally advanced rectal cancer by adding hydroxychloroquine to the standard preoperative regimen of 5-FU and radiation therapy.

Terminology

Autophagy is an evolutionarily conserved, self-digestive process in which proteins and other cytoplasmic material are recycled to support cell survival under stressful conditions (*i.e.*, cancer therapy).

Peer review

This manuscript addresses an important research question - Whether autophagy inhibitor can enhance the radio-sensitivity in treating locally advanced rectal cancer. The experimental design regarding outcome measures chosen seems well considered.

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REVIEW

Use of blood-based biomarkers for early diagnosis and surveillance of colorectal cancer

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Abstract

Early screening for colorectal cancer (CRC) holds the key to combat and control the increasing global burden of CRC morbidity and mortality. However, the current available screening modalities are severely inadequate because of their high cost and cumbersome preparatory procedures that ultimately lead to a low participation rate. People simply do not like to have colonoscopies. It would be ideal, therefore, to develop an alternative modality based on blood biomarkers as the first line screening test. This will allow for the differentiation of the general population from high risk individuals. Colonoscopy would then become the secondary test, to further screen the high risk segment of the population. This will encourage participation and therefore help to reach the goal of early detection and thereby reduce the anticipated increasing global CRC incidence rate. A blood-based screening test is an appealing alternative as it is non-invasive and poses minimal risk to patients. It is easy to perform, can be repeated at shorter intervals, and therefore would likely lead to a much higher participation rate. This review surveys various blood-based test strategies currently under investigation, discusses the potency of what is available, and assesses how new technology may contribute to future test design.

Key words: Colorectal neoplasms; Early detection of cancer; Colonoscopy; Biological markers; Blood; Messenger RNA; MicroRNA; Long non-coding RNA; DNA methylation; Microsatellite instability; Loss of heterozygosity; High-throughput nucleotide sequencing; Mass spectrometry; Real-time polymerase chain reaction; Microarray analysis

Core tip: Current colorectal cancer screening modalities are severely inadequate for global application because of high costs and a low participation rate. The alternative is to develop a blood-based screening test based on biomarkers which can replace colonoscopy as a first-line screening tool. The blood-based test should identify the high risk population, which will then be followed by colonoscopy as a secondary test. This review surveys the various experimental approaches and latest research into ideal biomarkers for the initial screening test, the pros and cons of each method and their potential to lead to a future screening test.

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INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer and fourth most common cause of cancer death in the world[1]. It is anticipated that as global communities become more developed and the world population ages, the morbidity and mortality rates due to CRC will increase substantially[2]. Although a number of early detection screening modalities have been used extensively in developed nations to lower the incidence and mortality rate, their overall high cost and low participation rate render them to be ineffective in controlling CRC on the global scale. Therefore, an alternative first line screening modality that has high sensitivity, high specificity, is relatively inexpensive and easily implemented, is urgently needed to help reduce the expected increase in global CRC burden. The main purpose of this review is to investigate the potential application of blood-based biomarkers in early diagnosis and surveillance of CRC cases.

URGENT NEED FOR A NEW CRC SCREENING MODALITY

Cancer is the leading cause of death in countries with a very high human development index and is poised to become a major cause of morbidity and mortality in every region of the world in the next few decades[3]. The United Nations has forecasted that the global population will reach 7.2 billion by July 2013, but population growth will slow in the next few decades, reaching 9.6 billion in 2050 and 10.9 billion in 2100 according to the medium-variant projection[4]. The United Nations report further delineated that the population growth will trend toward a balance between declining fertility rate and increasing population longevity. The increase in the aged population is expected to translate into an increasing global burden of cancer incidence[3,5]. In particular, it is anticipated that when the global population as a whole becomes more developed through rapid societal and economic changes, infection-related cancers (*i.e.,* cervical, stomach and liver cancers) will continue to decline but will be replaced with an increasing number of cancers associated with reproductive, dietary, and hormonal factors (*i.e.,* breast, colorectal, lung, and prostate cancers) as is typically found in high human development index regions.

Therefore, it is crucial to develop an early diagnostic modality for CRC that can be adaptable, economical, and implemented en masse by the global community.

Current screening options and their pros and cons

In the United States, CRC is the third most common cancer diagnosed among men and women and the second leading cause of cancer death with the estimation of 142280 new cases and 50830 deaths in 2013[6]. The five-year survival rate is 90% for cancer found localized or confined to the bowel wall, 70% for cancer with lymph node involvement, and 10% for cancer that has metastasized. Clearly, these numbers demonstrate that screening and early detection would lead to better survival, prognosis, treatment options, and hence quality of life. In 1980, the American Cancer Society (ACS) issued a formal guideline for CRC screening in average-risk adults, including an annual digital rectal exam and stool guaiac slide test in addition to the performance of a sigmoidoscopy every three to five years[7]. Since the guideline was issued, the cancer morbidity and mortality rates, which peaked around 1985 in the United States, have been in steady decline[6]. It is conceivable that the decline of CRC rates is at least partially attributable to the implementation of early screening and surveillance programs[8].

As of 2008, the basic screening modalities remain remarkably similar to those used in 1980 when the original guideline was issued, even when taking into account the development of newer technology in subsequent years[8]. In general, ACS, American College of Radiology (ACR), and the United States Preventive Services Task Force (USPSTF)[9] all agree on and emphasize the importance of CRC screening[8,10-12]. The recommended CRC screening modalities can be roughly divided into two different categories: fecal tests and direct structural exams.

The fecal tests are essentially "blood in the stool" tests. They can be performed using either a hemoglobin test [the guaiac-based Fecal Occult Blood Test (gFOBT)] or a newer and more sensitive version of an antibody-based globin test, known as the immunochemical FOBT or Fecal Immunochemical Test (FIT)[13]. In general, the gFOBT test is a non-invasive, inexpensive and easily applicable screening test which patients can readily perform in the comfort of their own home. Specimens from a FIT must be submitted to a laboratory for testing. The fecal tests help to reduce the risk of CRC death but has no effect on all-cause mortality[14]. They are not specific tests for CRC markers, and if found positive, the presence of CRC must still be confirmed by a direct structural exam such as colonoscopy or imaging procedures[15]. The fecal tests have high false positive rate for detecting CRC as gastrointestinal bleeding may occur in other conditions like colitis and hemorrhoids[16-18]. This, therefore, increases the burden of unnecessary colonoscopies and anxiety among patients[19]. It also may not detect precancerous lesions or early stage adenomas as bleeding may not be readily detectable in the presence of these conditions[20,21]. Regarding the fecal tests in general, the opportunity for CRC prevention is both limited and incidental and they are therefore not recommended as the solo screening test for CRC[8].

Direct structural exams include endoscopic procedures, such as flexible sigmoidoscopy and colonoscopy, and imaging procedures, such as double-contrast barium enema and computed tomographic colonography. In general, both flexible sigmoidoscopy and colonoscopy are invasive procedures using a colonoscope. Sigmoidoscopy is a small-scale colonoscopy which can be performed with a simple preparation without sedation, and

is used to examine the lower half of the colon lumen as opposed to the entire colon. The complete colonoscopy allows direct mucosal inspection of the entire colon from the appendiceal orifice to the dentate line. Same-session biopsy sampling or definitive treatment by polypectomy in the case of precancerous polyps and some early-stage cancers can also be performed. The double-contrast barium enema and computed tomographic colonography are both imaging examinations of the colon in its entirety and are either noninvasive or minimally invasive. However, although they allow for complete examination of the colon, there is no opportunity for biopsy or polypectomy and must therefore be followed up by therapeutic colonoscopy when polyps are found.

Inadequacy of colonoscopy

In the United States, colonoscopy has become the gold standard of CRC screening. It is one of the critical screening procedures recommended by ACS, ACR, and USPSTF, and it is also recommended by the American College of Gastroenterology as the preferred screening test[22]. The principal benefit of colonoscopy is that it allows for a full structural examination of the colon and rectum in a single session and for the detection of colorectal polyps and cancers accompanied by biopsy or polypectomy. Therefore, it has been performed with much higher frequency than all other procedures[23].

However, even in the United States where the technology and procedure are widely available, the colorectal screening participation is still low among average-risk adults in the range of 29.8% to 55.2%[24]. The participation rate is also surprisingly low at 40% for people at increased risk of CRC[25,26]. The majority of United States adults are not receiving regular age- and risk-appropriate screening due to concerns of cost, risk, and the discomfort and cumbersome preparation associated with the procedure[27-29]. The same is true in other European and Asian nations[2,30-32].

Although colonoscopy is the most effective screening method for CRC, there are various reported risks associated with the procedure, including bleeding (1.64 per 1000 patients), perforation (0.85 per 1000), death (0.074 per 1000), missed adenoma (6%-12%), and missed cancer (5%)[33]. The observed rate of missed polyps and/or cancer are largely due to variations in polyp size and other factors such as sub-optimal bowel preparation, experience of the endoscopists, and patient anatomical variations[34]. When it is taken into consideration that the guideline for the average-risk adult is to undergo colonoscopy every 10 years beginning at age 50[8,22] coupled with the rate of missed polyps being between 6% and 12%, there is still risk of developing CRC even when regular colonoscopy screening guidelines are followed.

Importance of an alternative screening method for CRC

The goals of any test are to detect disease early, improve duration and quality of life, reduce mortality and/or morbidity, and augment patient participation for that disease process-all at a very low risk and cost. To this end, the current CRC screening modality based on colonoscopy is severely inadequate. Despite all of the benefits that colonoscopy can offer as a screening procedure for CRC, concerns about its cost, risks, cumbersome preparatory procedure, and willingness of the general public to participate seriously compromise its effect in undermining the global CRC burden[35-37].

In an ideal world, the first line screening should be performed to identify a high risk segment of the population and then use a more extensive test (colonoscopy) on this sub group to reduce incidence of advanced diseases. In other words, it is crucial for the first line screening program to separate the following three entities: the general population (average risk), high risk group, and cancer group. Despite its non-specific nature, the simple FIT, when coupled with colonoscopy, has helped to dramatically reduce cancer incidence and number of deaths - In 100000 average risk patients, this screening has helped to reduce the number of cancer cases from 4875 to 1393, and number of cancer deaths from 1782 to 457[38]. Therefore, a more effective and sensitive blood-based biomarker test, supported by evidence from larger studies with solid results, can readily replace the stool-based test.

In order to establish a screening test, it must be evaluated for the following elements: frequency of performance, risk of complications, limitations, and false positive and negative rates. A blood-based test could be ideally used as a first line screening if all these elements were reliably determined and optimized. Colonoscopy would then become the secondary test, not the primary one. There will be greater willingness, by physicians and patients alike, to perform a blood test every several years than to justify the bowel preparation and complications of colonoscopy every 5-10 years.

BLOOD-BASED BIOMARKER FOR SCREENING CRC

Blood vessels are the human body's internal superhighways, for transporting nutrients to all cells in the body and carrying away waste products to avoid toxin buildup. Furthermore, they are also the body's chief communication channel into which signaling molecules such as hormones and cytokines are secreted and released in order to regulate a cascade of effector cell functions on distant sites. It would be ideal, therefore, to take advantage of this superhighway, with all of its abundant signaling molecules, to gauge a patient's health status.

The idea of a blood-based molecular test is appealing because the specimens can be obtained readily in a non-invasive manner, and it can be easily performed for any patient with minimal risk. If it were available, a blood-based test for CRC would reduce the overall cost, risk, and low patient participation issues that are typically associated with colonoscopy[39]. The key to developing a

useful blood-based molecular test is to find specific molecular indicators in the blood that are sensitive and specific for the detection of CRC. These indicators can be present in plasma or serum, and any form of molecules, including RNA, DNA, and protein[40-44].

Recent advances in the development of molecular diagnostic technology have allowed an expanding list of potential new screening modalities based on blood specimens to emerge. The available technologies, their current status, and their potential application will be discussed in further detail below.

Circulating RNA markers

RNA was originally thought to be highly labile, easily degradable, and therefore not likely to be stable or detectable outside of the protective cellular environment. However, numerous recent studies have shown that RNA are actually stable outside of cells[45,46], and all species of RNA, including both coding messenger RNA (mRNA)[47] and non-coding RNA, which includes microRNA (miRNA) and long non-coding RNA (lncRNA)[48,49], can be extracted and detected in the circulating blood plasma, serum, and other bodily fluids[50-52]. Furthermore, RNA expression is highly regulated in normal state but becomes increasingly dysregulated in a pathological state such as cancer[48,53]. Therefore, numerous studies have focused on profiling RNA expression, which may correspond to cancer state, and finding the indicator biomarkers for cancers[54-57].

mRNA markers

Various research groups have investigated the potential use of circulating mRNA as markers for cancer. The general experimental strategy is to employ microarray technology for mRNA expression profiling, which is then followed by validation using real time quantitative reverse transcription polymerase chain reaction (RT-qPCR). The specimens used are either mRNA extracted directly from blood serum/plasma or from peripheral blood cells[58]. Kopreski *et al*[47] demonstrated the possibility of detecting tumor mRNA, tyrosinase, in the serum of malignant melanoma patients although the result remains controversial[59]. Tsouma *et al*[60] extracted RNA from peripheral blood cells and used the multiplex RT-qPCR technology to determine the expression of three transcripts (carcinoembryonic antigen, cytokeratin 20 and epidermal growth factor receptor) to determine the disease stage and overall survival of CRC patients. DePrimo *et al*[61] and Twine *et al*[62] performed microarray-based mRNA expression profiling in peripheral blood mononuclear cells in 2003 and proposed some potential markers. However, this research generally remained at a proof-of-concept or pilot study stage, and further follow-up study has been sparse as the strategy they originally employed is now gradually being replaced by the new technology of Next Generation Sequencing (NGS), which will be discussed in more detail later.

ColonSentry as CRC screening or risk-assessment test?

Marshall *et al*[63] from GeneNews Ltd. developed a blood-based test using a seven-gene biomarker panel (*ANXA3, CLEC4D, LMNB1, PRRG4, TNFAIP6, VNN1* and *IL2RB*) testing RNA extracted from peripheral blood cells. This seven-gene panel was derived from a 196-gene expression profile using 112 CRC patients (including those with stage Ⅰ, Ⅱ, Ⅲ, and Ⅳ disease) and 120 controls. The panel was confirmed using 202 CRC patients (from all stages) and 208 controls, all from the Canadian population. They reported a sensitivity of 72% and specificity of 70% for this initial study. Then, they validated the seven-gene profile using 99 CRC patients (presumably from all stages) and 111 controls from the Malaysian population and reported 61% sensitivity and 77% specificity[64]. The researchers further validated their panel with an even larger population of 314 CRC patients (from all stages) and 328 controls from Canada and the United States, and they reported a sensitivity of 78% and specificity of 66%[65]. GeneNews now offers the ColonSentry test, presumably based on this seven-gene profile, as the world's first commercially available blood test for colon cancer screening, which is licensed to Enzo Clinical Labs of Enzo Biochem for the United States market. The test has recently been approved by the New York State Department of Health as a test to determine a person's risk of having CRC[66].

The ColonSentry molecular diagnostic test is marketed as a risk assessment tool rather than a cancer detection test. Although the experimental design for this seven-gene profile appeared to focus on identifying a pan-CRC marker panel when it profiled and validated a total of 727 CRC patients from all stages (estimated to be 30% stage Ⅰ, 30% stage Ⅱ, 30% stage Ⅲ, and 10% stage Ⅳ), there is no mention of any study on high risk individuals, advanced adenomas (AA), or patients with colon polyps that ultimately turned cancerous. It is therefore unclear how a set of pan-CRC markers for all CRC stages can be marketed as a risk assessment test. In any case, the test is considered experimental and investigational with many independent experts still questioning its effectiveness.

MiRNA as blood-based cancer markers

MiRNA are small non-coding RNA about 18-25 nucleotides in size[67]. A large body of publications indicates that miRNA regulate gene expression at the post-translational level in almost every biological event and play important roles in tumorigenesis, cancer development, migration and metastasis[68]. The differential expression of miRNA has been related to various cancers[69], and efforts have been made to profile the global and circulating miRNA expression patterns associated with various cancers, including breast cancer[70], lung cancer[71], lymphoma[72], ovarian cancer[73], and pancreatic cancer[74,75].

For CRC, studies have accumulated over the past five years that focus on profiling circulating blood plasma or serum miRNA and validating the findings with RT-qP-

CR. Ng *et al*[76] was the first group to profile 95 miRNA using a real-time PCR-based array on 5 CRC patients and 5 controls (presumably from the Chinese population in Hong Kong) and to validate the results with 90 CRC patients and 50 healthy controls. They identified miR-17-3p and miR-92 to be elevated significantly in CRC patients with 89% sensitivity and 70% specificity. Wang *et al*[77] profiled 742 miRNA using a miRNA microarray on 10 CRC patients and 10 normal controls from the Chinese population and validated the results with 90 CRC patients, 43 AA patients, and 58 healthy donors. They found miR-601 and miR-760 to be decreased in both CRC and AA patients when compared to healthy controls with 83.3% sensitivity and 69.1% specificity. Giráldez *et al*[78] performed a genome-wide profiling of 743 miRNA using a miRNA microarray on 21 CRC patients, 20 AA patients, and 20 healthy controls from the Spanish population, and they validated the findings using RT-qPCR with 42 CRC patients, 40 AA patients, and 53 controls. They identified a six-miRNA panel (miR-15b, miR-18a, miR-19a, miR-19b, miR-29a, and miR-335) as being able to differentiate CRC patients from healthy individuals with 78.57% sensitivity and 79.25% specificity, and miR-18a could also differentiate AA patients from healthy individuals with both 80% sensitivity and specificity. Luo *et al*[79] used a TaqMan MiRNA array to profile 667 miRNAs on 50 CRC patients and 50 controls from the German population and validated the results with new cohorts of 80 CRC patients compared to 144 controls and 50 AA patients compared to 50 controls. They identified nine miRNA (miR-18a, miR-20a, miR-21, miR-29a, miR-92a, miR-106b, miR-133a, miR-143, and miR145) to be differentially expressed in CRC patients and controls with the area under the accompanying receiver operating characteristic curve reported to be 0.745. The panel of miRNA did not, however, differentiate AA patients from the controls. Kanaan *et al*[80] screened for 380 miRNA using microfluidic TaqMan array technology on 20 CRC patients, 9 AA patients (referred to as colorectal adenomas), and 12 healthy donors of mixed racial background in the United States. They then validated the findings with a new cohort of 45 CRC patients, 16 AA patients, and 26 healthy controls; they derived an eight-miRNA panel (miR-15b, miR-17, miR-142-3p, miR-195, miR-331, miR-532-5p and 532-3p, and miR-652) that can distinguish AA patients from controls with 88% sensitivity and 64% specificity, and a three-miRNA panel (miR-431, miR-15b, and miR-139-3p) to differentiate stage Ⅳ CRC patients from controls with 93% sensitivity and 74% specificity. Ahmed *et al*[81] performed a profiling using miRNA microarray chips covering miRNA based on the published miRBase v17 list (presumed to be 1733 human miRNA) and validated their results using TaqMan RT-qPCR to analyze a panel of miRNA expression both in CRC patient plasma and tissues. They found nine miRNA (miR-7, miR-17-3p, miR-20a, miR-21, miR-92a, miR-96, miR-183, miR-196a and miR-214) to have increased expression and six miRNA (miR-124, miR-127-3p, miR-138, miR-143, miR-146a, and miR-222) to have reduced expression in both CRC patient plasma and tissues with 90% sensitivity and 95% specificity.

A few studies selected their miRNA markers based on published literature and re-confirmed the results with RT-qPCR assays. Huang *et al*[82] measured the levels of twelve miRNAs (miR-17-3p, -25, -29a, -92a, -134, -146a, -181d, -191, -221, -222, -223, and -320a) studied in the literature in 120 CRC patients, 37 AA patients, and 59 healthy controls from the Chinese population, and they confirmed miR-29a and miR-92a as potential indicators for CRC with 83% sensitivity and 84.7% specificity. Similarly, Liu *et al*[83] measured the levels of five miRNAs (miR-18a, -21, -31, -92a, and -106a) in serum samples from 200 CRC patients, 50 AA patients, and 80 healthy controls from the Chinese population and identified miR-92a along with miR-21 to be both significantly higher in CRC patients with 68% sensitivity and 91.2% specificity. Pu *et al*[84] measured miRNA expression levels of three target miRNAs (miR-21, -221, and -222) in 103 CRC patients and 37 controls from the Chinese population and found elevated expression of miR-221 in CRC patients with 86% sensitivity and 41% specificity. Wang *et al*[85] screened three miRNAs (miR-29a, -92a, and -17-3p) in 38 metastatic CRC and 36 primary CRC patients, assumed to be from the Chinese population, but did not utilize healthy controls. They found miR-29a to be higher in CRC patients with liver metastases than in primary CRC patients with sensitivity and specificity of 75%, and hence miR-29a may be useful in discriminating metastatic from non-metastatic CRC patients. Cheng *et al*[86] screened three miRNAs (miR-21, -92, and -141) using a cohort of 102 CRC patients and an age-matched cohort of healthy donors of mixed racial background from the United States population, validated their findings using 156 CRC patients and matched controls from the Chinese population, and found miR-141 to be higher in cases of advanced CRC (stage Ⅳ) with 90.9% sensitivity and 77.1% specificity.

As summarized in Table 1, there are a total of 38 miRNA that have been studied and proposed as potential biomarkers for CRC in the publications discussed above. In general, most of these studies focused on early stage CRC patients while some also included borderline AA patients. When pooling from all the studies mentioned here, sensitivities in the range of 68%-91% were reported, but the majority (in 9 out of 12 cases) observed sensitivities in the 83%-91% range. Reported specificities were in the range of 41%-95%, but the majority (also in 9 out of 12 cases) were in the 70%-95% range. Some miRNA, including miR-15b, miR-17-3p, miR-18a, miR-20a, miR-21, miR-29a, and miR-92a, have been proposed by more than one group of investigators. One unique miRNA, miR-21, might actually be a useful pan-cancer marker as it is similarly up regulated in other cancers[87]. However, most of these studies have not yet been evalu-

Table 1 Potential blood microRNA markers

MiRNA	AA?	Ref.
Upregulated in primary CRC		
miR-7		[81]
miR-15b	√	[78,80]
miR-17-5p	√	[80]
miR-17-3p		[76,81]
miR-18a	√	[78,79]
miR-19a		[78]
miR-19b		[78]
miR-20a		[79,81]
miR-21		[79,81,83,87,160-163]
miR-29a		[78,79,82]
miR-92a		[76,79,81-83]
miR-96		[81]
miR-106b		[79]
miR-133a		[79]
miR-142-3p	√	[80]
miR-143		[79]
miR-145		[79]
miR-183		[81]
miR-195	√	[80]
miR-196a		[81]
miR-214		[81]
miR-221		[84]
miR-331	√	[80]
miR-335		[78]
miR-532-5p	√	[80]
miR-532-3p	√	[80]
miR-652	√	[80]
miR-1246		[164]
Upregulated in metastatic CRC		
miR-15b	√	[80]
miR-29a		[85]
miR-139-3p		[80]
miR-141		[86]
miR-431		[80]
Downregulated in primary CRC		
miR-124		[81]
miR-127-3p		[81]
miR-138		[81]
miR-143		[81]
miR-146a		[81]
miR-222		[81]
miR-601	√	[77]
miR-760	√	[77]

AA: Able to differentiate advanced adenoma; CRC: Colorectal cancer; MiRNA: MicroRNA.

ated beyond the proof-of-principle and pilot stage, and not all miRNA markers were subsequently studied and confirmed by other groups. For example, Faltejskova *et al*[88] was not able to confirm the potency of miR-17-3p, miR-29a, miR-92a, and miR-135b as biomarkers for CRC. Luo *et al*[79] and Ahmed *et al*[81] found differential miR-143 expression in their respective studies. Other potential markers such as miR-17-3p, miR-18a, miR-21, miR-92, and miR-221 were not confirmed in follow-up studies by other groups[82-84,86].

Clearly, it is comprehensible that different experimental designs, procedures and methods, endogenous controls, patient populations, instrumentation and lab personnel could contribute to the seemingly contradicting results that have been published thus far. Nevertheless, the 38 candidate miRNA markers together can be further investigated using currently available technology, such as the TaqMan RT-qPCR profile platform already utilized by some of the research groups. It is possible, therefore, to coordinate a multicenter clinical trial involving different research groups and incorporating patient populations from a wide variety of backgrounds. It would be critical to synchronize specimen collection, processing procedures, and storage conditions for the collected specimens. The experimental design should also be based on a coordinated and synchronized set of experimental procedures and instrumentation that utilize the same endogenous control(s). The validity of each of the 38 miRNA markers as a tool for diagnosing CRC can then be evaluated for their potential future application.

NEXT GENERATION SEQUENCING

Since the first drafts of the human genome were published in 2001, sequencing technology has advanced at an ever rapid pace[89]. The cost of sequencing has decreased from about $1000 per megabase of DNA sequence when the first generation Sanger-based sequencing machine was used in 2001, down to $0.1 per megabase of DNA sequence using the next generation sequencing machine in 2013[90,91]. The cost for personal whole-genome sequencing has dropped from $100000000 in 2001 to $4000 (sequencing offered by Illumina, Inc.) in 2013, and it could possibly be driven further down to $1000 in the imminent future[92]. The availability of the NGS has revolutionized biomarker studies[93]. It is now possible to perform direct RNA sequencing (RNA-seq)[94] to sequence the whole transcriptome, which includes the entire set of all RNA molecules-coding RNA (mRNA, rRNA, tRNA) and non-coding RNA (miRNA, lncRNA, and other small RNA species)[94,95].

RNA-seq is very versatile and has been used to analyze tissue RNA biomarkers in breast cancer[96], hepatocellular carcinoma[97], lymphoma[98,99], melanoma[100,101], and prostate cancer[100]. RNA-seq has also been used to analyze gene expression signatures associated with survival[100], smoking status[102], and altered expression associated with *KRAS* mutation[103] in lung cancer. In terms of CRC, Wu *et al*[104] have performed transcriptome profiling comparing CRC, adjacent normal, and distant normal tissues and have identified 5 differentially expressed genes, including *ITGB5, COL1A1, FN1, SPP1,* and *COL3A1*, as well as alternative splicing, isoforms, and gene fusion events. It is anticipated that with the ability to extract and sequence RNA from blood plasma, more studies on blood-based RNA markers, based on RNA-seq technology, will soon emerge.

lncRNA markers

Given the increased availability of RNA-seq technology, it is now possible to study the lncRNA, which was dismissed as "junk" in the past but has now been found to regulate gene expression and cellular functions[105].

LncRNA, like its miRNA counterpart, plays major roles in tumor suppression and oncogenic functions and has been found to be dysregulated in human cancers[106]. Therefore, its potential role as biomarkers for cancer and other diseases has been investigated extensively[107,108]. As an example, Prostate cancer antigen (PCA3, also known as DD3) is a non-coding RNA that is highly sensitive and now used as a biomarker for the urine diagnostic test of prostate cancer[109-111].

In terms of CRC, research is currently focused on the role of lncRNA as tissue biomarkers. Ge *et al*[112] found that Prostate cancer-associated ncRNA transcripts 1 was upregulated in CRC tissue but not in adjacent normal tissue. Zhai *et al*[113] found that long intergenic noncoding RNA-p21 was upregulated in CRC tissue, and the expression level seemingly correlated with tumor progression (higher expression in later stages). Ling *et al*[114] showed a novel lncRNA-CCAT2 was highly overexpressed in CRC, and it was shown to be promoting tumor growth, metastasis and chromosomal instability. Kogo *et al*[115] demonstrated that expression of lncRNA-HOTAIR, which is known to reprogram chromatin organization and promote breast cancer metastasis[116], is also higher in stage Ⅳ CRC patients with liver metastases. Xu *et al*[117] found the lncRNA-human metastasis associated lung adenocarcinoma transcript 1 (MALAT-1) to be dysregulated in cancer, and the mutation on the 3' end of MALAT-1 is apparently tumorigenic. It is conceivable that RNA-seq technology can help facilitate further investigation into lncRNA functions and exploration of blood-circulating lncRNA as potential biomarkers for CRC and other cancers in the future.

BLOOD-BASED CIRCULATING DNA MARKERS

The presence of tumor DNA in circulating blood (plasma or serum) has been documented dating back to 1977[118]. Cell-free DNA (cfDNA) was thought to be released from either apoptotic or necrotic cancer cells, from direct secretion or as a byproduct of phagocytosis from macrophages or other scavenger cells[119,120]. Originally, it received little attention, but with recent advances in next generation sequencing (NGS) technology, it has been explored extensively for the potential application to cancer detection[121]. In general, the studies of cfDNA as cancer biomarkers focus on monitoring the presence of promoter hypermethylation, aberrant tumor DNA mutation, microsatellite alternations, and mitochondria DNA in blood circulation. The validity of each approach will be discussed below.

Aberrant DNA methylation as markers

Aberrant DNA methylation has been associated with tumorigenesis as a consequence of the alteration it causes in gene expression[122,123]. For example, hypermethylation of tumor suppressor promoter genes would cause inappropriate gene silencing and therefore lead to cancer[124]. In general, DNA methylation is thought to be associated with an early event in tumorigenesis and has therefore been proposed as a potential early cancer detection marker[123,125]. The research strategy typically focuses on using methylation specific PCR (MSP) to study hypermethylation of methylation sites, in CpG dinucleotides or in CpG islands, in the promoters of tumor suppressor genes[124,126]. In the context of CRC, Nakayama *et al*[127] and Lecomte *et al*[128] both monitored the hypermethylation of the promoter of tumor suppressor gene *p16* and found the plasma in 21 of 31 (68%) patients and 31 of 45 (69%) patients, respectively, to be positive. Grady *et al*[129] found aberrant hypermethylation of the human MutL homolog 1 (*hMLH1*) promoter in the sera of 9 out of 19 (47%) cases of CRC. Leung *et al*[130] monitored promoter hypermethylation in three genes, adenomatous polyposis coli (*APC*), *hMLH1,* and *helicase-like transcription factor,* and found at least one of the three genes with methylated promoter DNA in the sera of 28 out of 49 (57%) CRC patients. Additional genes monitored for tumor-related promoter hypermethylation, including the putative metastasis suppressor gene *death-associated protein kinase*, the detoxification gene *glutathione S-transferase P1*, the DNA repair gene *O*^{6}*-methylguanine-DNA-methyltransferase,* and *p14-ARF* in other cancers exhibit a detection rate that is generally in the range of 42% to 73%[131-133]. It is conceivable that NGS technology can be coupled with MSP to identify a pool of tumor suppressing genes silenced in association with early stage CRC and AA, test their corresponding promoter methylation, and generate a set of candidate markers based on epigenetic changes as a screening panel for CRC in the future.

Aberrant tumor DNA mutation markers

The NGS technology has been employed for somatic mutation analysis in CRC[134], particularly on several high mutation frequency genes, such as *K-RAS*[128,135,136], *TP53*[137], and *APC*[138]. However, the percentage of circulating tumor DNA is relatively low when compared to wild-type DNA[139]. For example, Diehl *et al*[138] has shown that in advanced CRC, the mutated *APC* DNA fragment is found to be in the range of 1.9% to 27% of cfDNA but only 0.01% to 0.12% in early stage CRC. Even with direct sequencing technology, it does not allow reliable detection of less than 25% mutant signal in a background of wild-type DNA[140]. Furthermore, the tumor-associated mutations are often unique with each patient[141,142], and therefore, based on the current available technology, it is less likely to develop a low cost and highly sensitive comprehensive test to cover all somatic mutations for early cancer detection.

Microsatellite alterations as markers

Microsatellite alterations, which include microsatellite instability (MSI) and loss of heterozygosity (LOH), are known to be associated with tumorigenesis and cancer

progression and therefore were proposed as potential tumor markers detectable in cfDNA[143]. MSI analysis focuses on measuring the specific polymorphic tetranucleotide repeat and/or dinucleotide markers that are located in regions frequently shifted or altered in cancer, and LOH analysis focuses on the loss of specific chromosomal regions bearing tumor suppressors. Hibi *et al*[141] examined microsatellite alterations and found LOH or microsatellite shift of at least one locus (18a, 17p, and 8p) in 35 of 44 (80%) primary CRC tumors, but none of the LOH or microsatellite shifts were detected in the corresponding serum DNA. Several other groups focused on different cancers with most success in metastatic cancers[143,144]. In general, microsatellite alteration analysis exhibits relatively low sensitivity and specificity in detecting early stage cancer.

Circulating mitochondrial DNA as markers

There are generally a few hundreds of copies of mitochondrial DNA in each cell[145]. Due to its multi-copy nature, mtDNA is frequently found to be heteroplasmic, with a heterogeneous mixture of polymorphic variants. In cancer cells, mtDNA harbor further heteroplasmic alterations associated specifically with cancer, especially in the highly variable D-loop (displacement loop) region. With the NGS, the approaches generally focused on either differential copy number of mtDNA versus gDNA, or mtDNA alteration and tumor-associated mtDNA mutations[146]. For CRC, Hibi *et al*[147] has studied mtDNA alternation in early CRC patients and found that 7 out of 77 (9%) CRC tissues contained true somatic mutations in the D-loop region, but only one out of these 7 positive patients (14%) were noted to have mtDNA alterations in their serum DNA. Due to of the relatively low detection rate of early stage cancer, most studies therefore focused on its potential application in metastatic cancers[148-153].

IDENTIFICATION OF BLOOD-BASED PROTEIN MARKERS

The study of blood-based protein markers in general focuses on proteins secreted, shed or leaking from cancer cells into the blood stream. This is generally referred to as "cancer secretome"[154]. The cancer secretome can be studied comprehensively by several mass spectrometric technologies. Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) and HPLC-electrospray ionization mass spectrometry (ESI-MS) analyze biomolecules in biological fluids[155,156]. Surface-enhanced laser desorption ionization-time of flight mass spectrometry (SELDI-TOF MS) can be used as a serum protein profiler to identify new biomarkers[157]. Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) can fractionate and identify the specific molecules of interest[154]. There is also an Aptamer proteomic technology that can be used to identify biomarkers for cancer[158]. Many candidate protein biomarkers have been generated based on these technologies.

However, the application of these technologies remains research-oriented. The potency of their translational capability in clinical and diagnostic application requires further investigation[159].

CONCLUSION

Early screening of CRC is clearly the most effective way to combat the anticipated increase of global CRC morbidity and mortality. Despite all recent technological advances, the currently available screening modalities remain archaically similar to 33 years ago. The most effective screening modality today is through the invasive procedure of colonoscopy. However, even in the United States, where the procedure is widely available and publicized, covered by most medical insurance plans, and recommended by medical professionals and practitioners, the participation rate is still pathetically low. It is conceivable that the participation rate would not fare better even if it were widely available on a global scale. Clearly, a new first line CRC screening procedure that is inexpensive, low risk, highly sensitive, and does not require cumbersome preparation is desirable.

A blood-based screening test for CRC would be an attractive alternative to colonoscopy if it were available because it is essentially non-invasive and relatively painless to the patient. Ideally, a blood-based test can be a useful first line screening tool for the general population at average risk, thereby separating out high risk and CRC patient groups. However, for patients with known high risk factors, including family history of CRC, familial adenomatous polyposis, hereditary nonpolyposis CRC, inflammatory bowel disease, history of polyps, or previous CRC, colonoscopy should still be the primary method of screening and follow-up starting at age 50, although a blood-based test can still be used for screening these patients earlier at age 40. In short, circumstances under which a blood-based screening test is used should be determined based on the sensitivity and specificity of the methodology developed in the future.

The key to establishing a good blood-based test is to find highly sensitive and specific biomarkers in the blood. As discussed in this review, various types of biomarkers have been proposed and explored by many research groups to varying degrees. Table 2 summarizes the sensitivity, specificity, and estimated cost for the types of stool-based tests, structural exams, and potential blood-based tests as discussed in this review. The ColonSentry® seven-gene mRNA biomarker panel is the first commercially available blood test that is supposed to determine the risk of developing CRC. The sensitivity and specificity for this "risk assessment" are 78% and 66% respectively. As shown in Table 2, among all the biomarker types, the miRNA markers demonstrated the greatest potential because most publications reported a relatively high sensitivity (83%-91%) and specificity (70%-95%) rate, utilized mostly AA and early stage CRC patient, and

Table 2 Comparison of colorectal cancer screening tests

Test name	Cost	Procedure type	Prep?	Sensitivity	Specificity	Note	Ref.
gFOBT	$5[3]	Stool test	Yes[1]	12%[2] and 40%	98%	Hemoccult Ⅱ	[165]
iFOBT/FIT	$22[3]	Stool test	Yes[1]	22%[2] and 70%	95%		[165]
Fx. Sigmoidoscopy	$500-$750[3]	Invasive	Yes	95%[2] and 95%	92%		[165]
Colonoscopy	$800-$1600[3]	Invasive	Yes	95%[2] and 98%	90%		[165,166]
DCBE	$250-$500[3]	X-ray	Yes	48%[2]	90%	Not recommended by USPSTF	[166]
CTC	$400-$800[3]	CT-scan	Yes	59%[2]	96%	Not recommended by USPSTF	[166]
Blood-based test							
ColonSentry®	$350	blood-test	No	78%	66%	GeneNews/Enzo Biochem	[66]
MiRNA (5-gene)	Est. $250[4]	blood-test	No	Est. 83%-91%	Est. 70%-95%		
LncRNA (1-gene)	$385.00[5]	blood-test	No	N/A	N/A		
DNA methylation	Est. $250[4]	blood-test	No	Est. 42%-73%	Est. 42%-73%		

[1]Required to clean colon; [2]For detecting advanced adenoma at ⩾ 10 mm; [3]Cost estimated from Colon Cancer Alliance website (http://www.ccalliance.org/index.html); [4]Cost estimated based on The Valley Hospital Histology Lab charge; [5]Cost estimated based on PCA3 test offered by GD Specialized Diagnostics. Fx. Sigmoidoscopy: Flexible Sigmoidoscopy; DCBE: Double-contrast barium enema; CTC: Computed tomographic colonography; USPSTF: United States Preventive Services Task Force; gFOBT: Guaiac-based Fecal Occult Blood Test; iFOBT: Immunochemical Fecal Occult Blood Test; FIT: Fecal Immunochemical Test; MiRNA: MicroRNA; LncRNA: Long non-coding RNA; CT: Computed tomography; N/A: Data not available; PCA3: Prostate cancer antigen 3.

studied a wide variety of patient populations. Therefore, a multi-center clinical trial with synchronized experimental procedures that tested all 38 miRNA listed in Table 1 could be considered. On the other hand, the aberrant DNA methylation analyses on promoters of tumor suppressors also demonstrated a high potential to be developed into a cancer screening test. With available NGS technology and MSP showing relatively high sensitivity and specificity (42%-73%), it is now possible to explore more tumor-specific promoters, which might have higher sensitivity and specificity and eventually be developed into a screening test.

On the other hand, although research studies of lncRNA markers using NGS are still at the early stage, it has a great potential to be developed into a CRC screening test as well. It is especially encouraging to see one of the lncRNA, PCA3, is now used routinely as a prognostic marker for prostate cancer. With the wider availability of NGS, it is anticipated that more studies will be undertaken to generate new candidate genes and biomarkers, which would possibly lead to a future diagnostic test for CRC.

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BRIEF ARTICLE

Colorectal carcinoma in a Southern Mediterranean country: The Libyan scenario

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Abstract

AIM: To study the salient features of colorectal cancer (CRC) in Libya.

METHODS: Patients records were gathered at the primary oncology clinic in eastern Libya for the period of one calendar year (2012). Using this data, various parameters were analyzed and age-standardized incidence rates were determined using the direct method and the standard population.

RESULTS: During 2012, 174 patients were diagnosed with CRC, 51.7% (n = 90) male and 48.3% (n = 84) females. The average age was 58.7 (± 13.4) years, with men around 57.3 (± 13) years old and women usually 60.1 (± 13.8) years of age. Libya has the highest rate of CRC in North Africa, with an incidence closer to the European figures. The age-standardized rate for CRC was 17.5 and 17.2/100000 for males and females respectively. It was the second most common cancer, forming 19% of malignancies, with fluctuation in ranking and incidence in different cities/villages. Increasingly, younger ages are being afflicted and a higher proportion of patients are among the > 40 years subset. Nearly two-thirds presented at either stage Ⅲ (22.4%) or Ⅳ (38.4%).

CONCLUSION: Cancer surveillance systems should be established in order to effectively monitor the situation. Likewise, screening programs are invaluable in the Libyan scenario given the predominance of sporadic cases.

Key words: Colorectal carcinoma; Cancer incidence; Age-standardized rates; Benghazi, Libya; North Africa; Young age; Urban-rural differences

Core tip: Colorectal cancer incidence in Libya has changed greatly since the last time it was determined nearly a decade ago. Libya was found to have the highest incidence rate in North Africa, with younger ages more affected. Late presentation was found to be a major problem in the Libyan case. Clear urban-rural differences were seen when the different districts were analyzed. Different hypotheses are put forth to explain these variations. Proper surveillance and screening programs need to be established and healthcare policies should be adjusted to take into account the increasing rate of this malignancy.

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INTRODUCTION

Colorectal cancer (CRC) is one of the most common

malignant tumors worldwide[1,2], with the disease incidence rising with advanced age[3,4]. The overall mortality from CRC is 60%, which represents the second leading cause of cancer death in western societies. Figures on incidence from Libyan sources are over a decade old and have multiple limitations[5]. Unfortunately, there has not been a major improvement in patient survival despite the advances made in our understanding of disease and in chemotherapy practice[6]. Surgical cure of CRC is determined by stage of the tumor and its biological behavior. Early CRCs can be cured with surgery alone.

Even today, most CRC patients undergo potentially curative surgery and receive adjuvant chemotherapy but approximately 50% of the patients initially thought to be cured subsequently relapse and die of their disease[7]. Advanced CRC is defined as a disease that is either metastatic or locally advanced and in which surgical resection is unlikely to be curative[8]. Once metastasis has occurred, the patient's prognosis is considerably worse, with the 5-year survival rate being < 5%[8]. For the majority of patients, chemotherapy can yield improvements in survival and is the main modality of treatment in these patients[9].

CRC was found to be the leading malignancy in Libyan males and the second most prevalent among females[10]. On a global scale, it is the third most common form of cancer[11].

On the whole, the incidence of colorectal carcinoma in Middle Eastern countries is lower than that of Western countries[12]. The North African countries have consistently contributed their registry data to scientific literature[13-18]. Due to a number of difficulties, very limited data exists for Libya[10,19,20]. Moreover, epidemiological features of CRC have never been studied, despite being a major form of malignancy. A unique research opportunity is offered in the Libyan scenario where the traditional lifestyle still prevails in rural areas and the urban (Westernized) mode of living dominates in the cities.

Using data that was actively collected from the Department of Oncology at the Benghazi Medical Center, the primary oncology center in eastern Libya, the salient features of colorectal carcinoma patients were analyzed.

MATERIALS AND METHODS

Study population

Libya is a North African country categorized under the Eastern Mediterranean Regional Office in the WHO classification. According to the 2006 census, over 5.5 million people lived in Libya, with 28.5% (n = 1613749) residing in the eastern part of the country. Benghazi is the largest city in eastern Libya, with over 670000 inhabitants. The catchment area includes eight major locations comprising urban, suburban and rural populations (Figure 1) and patients were classified under these main districts according to proximity.

Ethical approval

The study was approved by the Biomedical Ethics Committee at the Libyan International Medical University. All personal identifiers were stripped from the data and only medically significant data was analyzed.

Figure 1 Map of Libya highlighting the districts that were studied and included in the eastern Libya cancer pool.

Data collection

Data was obtained from the patient records at the Department of Oncology in the Benghazi Medical Center who were diagnosed from the period of January 1st to December 31st, 2012. In Libya, an ineffective primary health system forces the populace to deal directly with outpatient departments in secondary and tertiary centers. This is true for Libyan oncology patients where they all present to the oncological outpatient department after a referral from another specialty. They are then diagnosed and given a treatment plan. The department effectively receives all the cancer cases in Benghazi and the overwhelming majority of the cases in eastern Libya (being the only oncological center in the region). The patients were diagnosed through various techniques, particularly microscopic verification and clinically/radiologically diagnosis. However, due to clerical difficulties, this parameter (*i.e.*, the method of diagnosis) could not reliably be collected for all patients and hence was excluded from the analysis. This data serves as a good indicator for eastern Libya in general and Benghazi in particular.

Hematological malignancies were not included in this study since such patients are recorded at the Department of Hematology and their data was not made available.

Different parameters were recorded for each patient: age, gender, city, type of cancer, subtype and staging. In the light of clerical errors, a number of cases were set aside for a certain parameter but used for others. The patients were filtered by city of origin to include only patients residing in the eastern part and not referrals.

Statistical analysis

The data was computerized in a data sheet and organized as per International Classification of Diseases for Oncol-

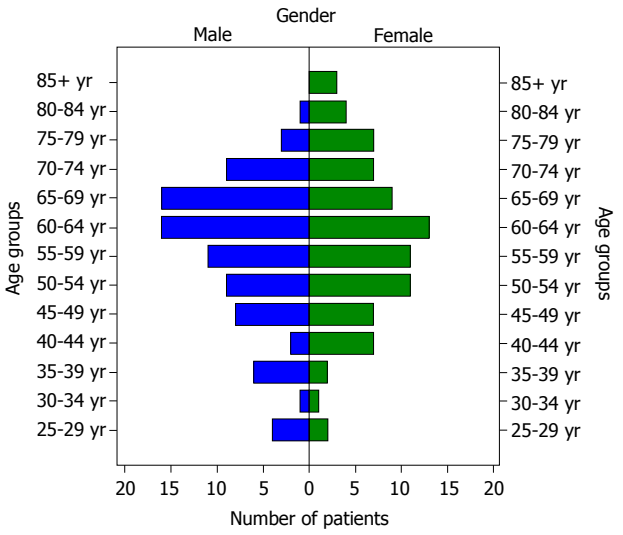

Figure 2 Population pyramid of the colorectal cancer patients split by gender.

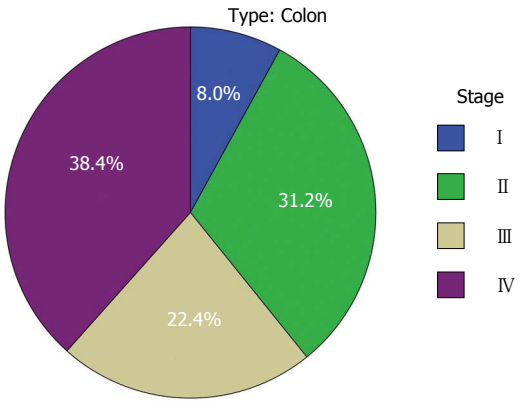

Figure 3 Distribution of colorectal cancer patients according to clinical stage at diagnosis.

ogy (ICD-O). An SPSS-based model was designed that spanned the collected data and basic statistical procedures were performed (*t* tests and χ^2 tests).

The 2012 Libyan population was determined using the 2006 Libyan census, taking into consideration the appropriate population growth. Age-specific incidence and age-standardized rates (ASRs) were calculated *via* the direct method using the standard population distribution[15] arranged by site (ICD-O).

RESULTS

During 2012, a total of 174 patients were diagnosed with colorectal carcinoma in the eastern region of Libya. Slightly over half of the cases (51.7%, $n = 90$) were male, while 48.3% ($n = 84$) were females. The average overall age of the patients was 58.7 (± 13.4) years, with men around 57.3 (± 13) years old and women usually 60.1 (± 13.8) years of age. The ASR for CRC was 17.5 and 17.2/100000 for males and females respectively. It was the second most common cancer overall in the eastern region, forming 19% of all malignancies, with fluctuation in ranking in different towns/villages.

When the age was categorized into groups, it was found that a peak occurred in the 60-64 year age group (17.1%, $n = 29$), which was true for both genders. Nearly one tenth of colorectal carcinoma patients (9.4%, $n = 16$) were diagnosed < 40 years. Males were more than two-thirds (68.8%, $n = 11$) of these patients, giving a male to female ratio of 2.2. One quarter of CRC patients (23.5%, $n = 40$) presented before the age of 50 years and that figure jumped to over one-third of patients when cases under 55 years are studied (35.3%, $n = 60$). Figure 2 depicts the distribution of CRC by age and gender.

The three areas that contributed the greatest number of colon cancer cases were Benghazi (64.9%, $n = 113$), Al-Beida (9.8%, $n = 17$) and Al-Marj (8%, $n = 14$). When looking at population distribution from the Libyan 2006 census, one clearly observes that the city of Benghazi is over-represented, while the other (more rural) areas were starkly under-represented. Nearly two-thirds of colon cancer patients were from Benghazi, whereas its inhabitants constitute only 41% of the population in eastern Libya ($\chi^2 = 41.291$, $P < 0.001$). A small proportion (1.7%, $n = 3$) of the colon cancer patients were foreign nationals. The detailed classification and distribution of these parameters can be seen in Table 1.

Table 1 Display of key parameters of the cancer patients in eastern Libya

	Overall		Male		Female	
Age (*n*/SD)	58.7	13.4	57.3	13.0	60.1	13.8
Age group (*n*/%)						
20-29 yr	6	3.5	4	4.7	2	2.4
30-39 yr	10	5.9	7	8.1	3	3.6
40-49 yr	24	14.1	10	11.6	14	16.6
50-59 yr	42	24.7	20	23.3	22	26.2
60-69 yr	54	31.8	32	37.2	22	26.2
70-79 yr	26	15.3	12	14.0	14	16.6
80+ yr	8	4.7	1	1.1	7	8.4
Total	170	100.0	86	100.0	84	100.0
Nationality (*n*/%)						
Libyan	170	98.3	89	100.0	81	96.4
Non-Libyan	3	1.7	0	0.0	3	3.6
Total	173	100.0	89	100.0	84	100.0
City of origin (*n*/%)						
Ajdabia	8	4.6	6	6.7	2	2.4
Beida	17	9.8	9	10.0	8	9.5
Benghazi	113	64.9	56	62.2	57	67.9
Derna	6	3.4	3	3.3	3	3.6
Kufra	4	2.3	0	0.0	4	4.8
Marj	14	8.0	8	8.9	6	7.1
Tobruk	12	6.9	8	8.9	4	4.8
Total	174	100.0	90	100.0	84	100.0

The clinical stage was recorded for 125 patients (71.8%) and 49 were excluded due to clerical errors. The majority of cases (38.4%, $n = 48$) presented at stage Ⅳ with another 28 patients at stage Ⅲ (22.4%). This is further highlighted in Figure 3.

The cases were classified on the site of the cancer as being either right-sided or left-sided colorectal carcinoma. Cancers of the left colon were more common (78.6%,

Table 2 Distribution of the cases in terms of clinical staging, site of cancer and histopathological grade

	Overall		Male		Female	
Clinical stage (*n*/%)						
I -A	3	2.4	1	1.5	2	3.4
I -B	7	5.6	5	7.6	2	3.4
II -A	28	22.4	12	18.2	16	27.1
II -B	11	8.8	6	9.1	5	8.5
III -A	5	4.0	4	6.1	1	1.7
III -B	11	8.8	5	7.6	6	10.2
III -C	12	9.6	7	10.6	5	8.5
IV	48	38.4	26	39.4	22	37.3
Total	125	100.0	66	100.0	59	100.0
Site of cancer (*n*/%)						
Right side	30	21.4	14	18.9	16	24.2
Left side	110	78.6	60	81.1	50	75.8
Total	140	100.0	74	100.0	66	100.0
Histopathological grade (*n*/%)						
Well differentiated	29	33.3	17	35.4	12	30.8
Moderately differentiated	47	54.0	27	56.3	20	51.3
Poorly differentiated	11	12.6	4	8.3	7	17.9
Total	87	100.0	48	100.0	39	100.0

Table 3 The distribution of colorectal carcinoma based on site

Specific site	*n*	%
Anus	1	0.7
Appendix	1	0.7
Asc. colon	4	2.8
Cecum	6	4.2
Left side	25	17.5
Rectum	52	36.4
Right side	19	13.3
Sigmoid	35	24.5
Total	143	100.0

Table 4 Comparison of colorectal cancer incidence rates (age-adjusted per 10^5)

Country	Male	Female
Benghazi, Libya (2012)[1]	17.5	17.2
Benghazi, Libya (2003)[10]	11.6	8.8
Western Libya[11]	14.2	12.0
Algeria (Setif, 1998-2002)[6]	6.6	6.8
Algeria (Alger, 2006)[7]	14.8	11.0
Egypt (Gharbiah, 1999-2002)[6]	6.3	4.4
Tunisia (Sousse, 1998-2002)[6]	11.6	9.0
Tunisia (Sfax, 2000-2002)[9]	11.5	9.1
Morocco (Rabat, 2005)[4]	7.2	4.6
Morocco (Casablanca, 2004)[5]	6.6	5.7
European Pool (MECC)	22.0	15.6
Iran[15]	8.2	7.0

n = 110) than their right-sided counterparts (21.4%, *n* = 30). This is shown with other parameters in Table 2. When the specific sites were studied (*i.e.*, sigmoid, rectal, *etc.*), we found that rectal carcinomas were the most common form (36.4%, *n* = 52). This can be seen in Table 3.

Histopathologically, 87 patients (50%) had graded carcinomas. Most were moderately differentiated (54%, *n* = 47), followed by well differentiated (33.3%, *n* = 29) carcinomas; poorly differentiated cancers were the least common (12.6%, *n* = 11). This is further described in Table 2.

DISCUSSION

In terms of incidence, the average rate for Middle Eastern countries was reported as 3/100000-7/100000[21,22]. Even among the North African countries, eastern Libya claims the highest ASR for colon cancer (Table 4)[10,19,23]. While the exact reasons for this inordinately high rate remain to be ascertained, genetic predisposition, increased Westernization of the Libyan diet, physical inactivity and lack of screening programs may be considered important predisposing factors.

The distribution of colon cancer cases was fairly equal between the genders, despite a conflict in previous literature between reports supporting and others negating a difference between men and women. In terms of age, there was no significant difference between the genders (P = 0.072). The male to female ratio, skewed towards males in the < 40 years subset, was much higher than other nations[24].

Similarly to neighboring Egypt, younger age groups are affected with CRC[25]. One of the principle hypotheses for this trend in that the younger generation live a more Westernized lifestyle (*i.e.*, unhealthy diet with low exercise) and are hence at greater risk[26]. This is of particular importance since the prognosis proportionately worsens below the age of 40 years[27].

Benghazi is the largest city in eastern Libya and the second largest in all of Libya, with a population approaching 800000 inhabitants. Colon cancer was more common in the urban environment in Libya, potentially due to a more sedentary lifestyle, more Westernized diet and a subsequently higher prevalence of obesity. The rural areas in Libya have maintained a relatively traditional way of life with farming, animal rearing and small industries as the main occupations. Traditional cuisine focusing on whole grain and Mediterranean style meals is more common in that environment. While the urban-rural difference has been proven for breast cancer[28], the literature for colon cancer is scanty globally and virtually non-existent for the region.

Foreign nationals are less likely to present to the oncology clinic in Libya as they are more apt to return to their home countries and seek their family upon receiving such news. This would explain their small proportion in the sample.

Over 60% of patients presented at the oncology clinic at advanced stages (III/IV) when the long term prognosis is grim. Around 22.4% (*n* = 28) of our patients were diagnosed at stage III, while 38.4% (*n* = 48) presented at stage IV. This was found to be similar for other major forms of cancer studied in Libya[10]. The major problem in the Libyan scenario is late presentation. This could be due a number of different reasons, among them awareness and social stigma. Transport difficulties in rural areas

as well as the distance to Benghazi also serve as a hindrance to early detection.

Screening programs would greatly increase the catchment rate of our CRC patients before they reach these late stages. This is especially important in the sporadic cases, which form the majority of cases.

The Libyan diet is traditional in certain areas and modern (Westernized) in others. This is a reflection of the rural-urban differences that exist. With the increase of consumption of Western-style cooking and the downwards trend of traditional food, it is expected that there would be a rise in the incidence of CRC. However, a long term study is required in order to determine such a trend. Further risk factors also exist in Libyan society, such as a high rate of diabetes mellitus, smoking, obesity, *etc*.

Certain limitations, however, need to mentioned, namely the quality of the patient records. In the gathering of this data, not all the parameters were available for all the patients and hence they were excluded from the analysis. The data that was gathered for this study was from one center and, even although it is the sole oncological center in the region, there will surely be a certain number of missed cases or patients who immediately sought care abroad without referral to our center first. Additionally, while this data is representative of eastern Libya, we cannot generalize this for all of Libya. In cancer epidemiology, stark differences may exist between different regions of a country.

In conclusion, Libya has a higher rate of CRC than neighboring countries, with an incidence that is closer to the European figures. Increasingly, younger ages are being afflicted and a higher proportion of patients are among the > 40 years subset. Urban-rural differences were observed in the Libyan scenario. A major problem is delayed presentation with a large proportion of patients seeking medical care at advanced or late stages with a poor prognosis. Screening programs are sorely needed in Libya in order to combat presentation at late stages.

COMMENTS

Background

Cancer epidemiology is a rapidly growing field that has made great strides in the last few decades; however, it has always been developed countries that have contributed the majority of data and figures. As a consequence, most of the information available on cancer incidence is based on those societies. In the developing world, this information is extracted with more difficulty. This is especially true in Libya where data gathering is notoriously difficult (for a myriad of reasons). For the first time, colorectal cancer (CRC) patients in Libya were studied and the findings were presented.

Research frontiers

There is now a focus on customization of epidemiology for different countries and even different regions within a single country. Preventive medicine has taken the lead in epidemiology and a baseline needs to be determined before any cancer plan can be established at a national or local level.

Innovations and breakthroughs

Colon cancer was found to be the leading malignancy in Libyan males and the second most prevalent among females. Despite that, there has never been a study on CRC in Libya. Using population data from the 2006 Libyan census with projections for future years, the age-standardized rates (ASR) was calculated. Various parameters were gathered for the patients, among them, age, nationality, affected site within the colon, histopathological grade and the clinical stage. The geographical distribution of CRC patients in Libya was also studied for the first time.

Applications

Using the findings from this study, the health authorities in Libya can finally lay a plan to help combat CRC. A major problem in the Libyan scenario is late presentation, so increased awareness among the populace and a higher index of suspicion among clinicians would surely save countless lives. Certain regions contributed more in terms of patient load and hence more focus needs to be placed there.

Terminology

ASR: ASR is an internationally used measure of new cancer cases relative to the standard world population (as stated in the Cancer in Five Continents series).

Peer review

It is a descriptive study that intended to demonstrate the effect of changing food habits in Libyan people. This is an interesting article.

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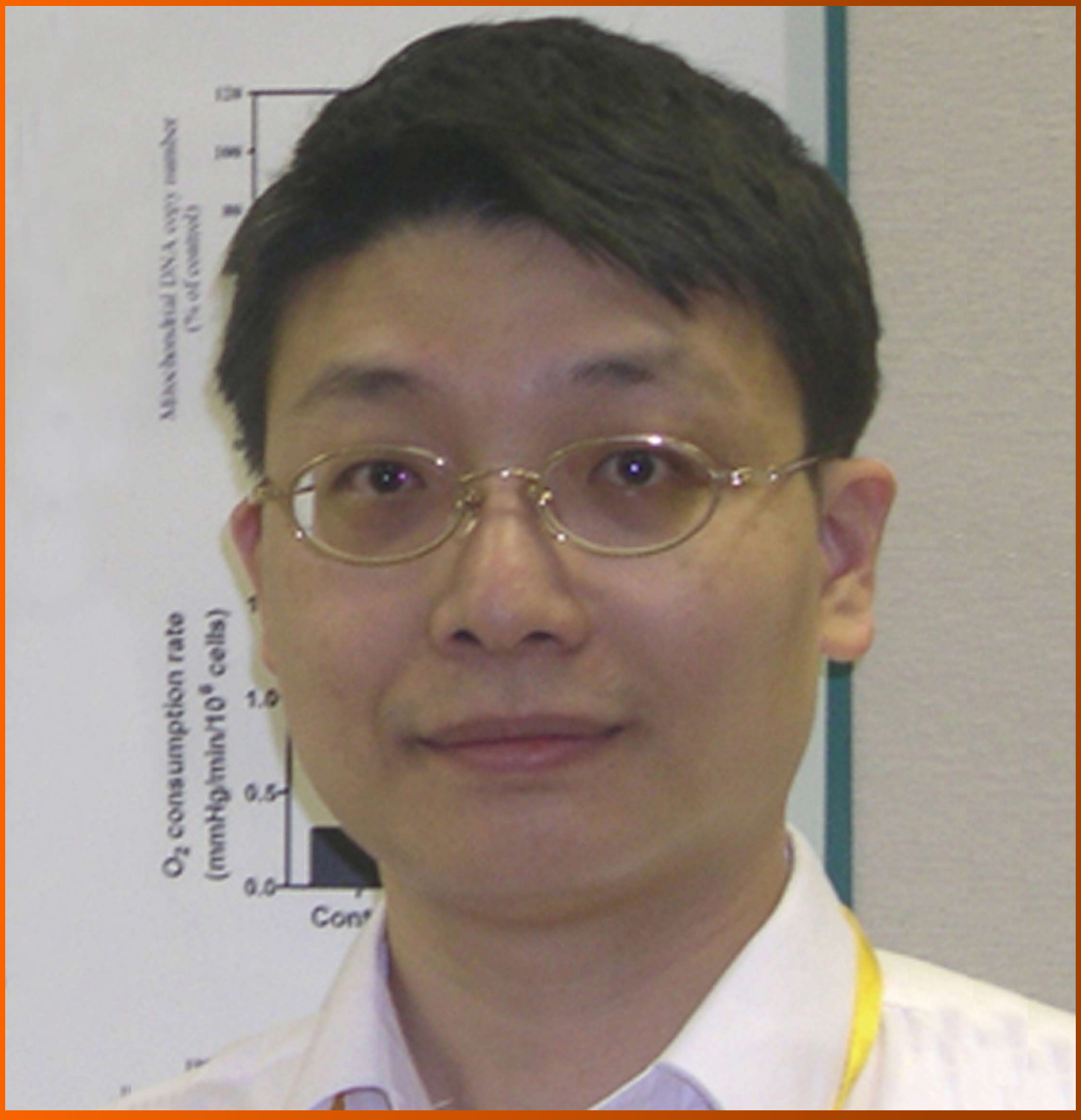

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REVIEW

Do the benefits outweigh the side effects of colorectal cancer surveillance? A systematic review

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Abstract

Most patients treated with curative intent for colorectal cancer (CRC) are included in a follow-up program involving periodic evaluations. The survival benefits of a follow-up program are well delineated, and previous meta-analyses have suggested an overall survival improvement of 5%-10% by intensive follow-up. However, in a recent randomized trial, there was no survival benefit when a minimal *vs* an intensive follow-up program was compared. Less is known about the potential side effects of follow-up. Well-known side effects of preventive programs are those of somatic complications caused by testing, negative psychological consequences of follow-up itself, and the downstream impact of false positive or false negative tests. Accordingly, the potential survival benefits of CRC follow-up must be weighed against these potential negatives. The present review compares the benefits and side effects of CRC follow-up, and we propose future areas for research.

Key words: Colorectal cancer; Follow-up; Surveillance; False positive; Cancer survivorship

Core tip: Most western countries have a national follow-up program for colorectal cancer (CRC) survivors. The reported reduction in absolute mortality from intensive follow-up is 5%-10%, though recent data from the follow-up after colorectal surgery randomized trial call this effect into question. There exists limited evidence of improved quality of life (QoL) due to participation in a follow-up program, and the impact of false positive tests on QoL might be considerable. Several national experts advocate for low-cost, low-intensity CRC follow-up programs.

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INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer in the western world, and surgery is the only curative treatment. Approximately one-third of those surgically resected will experience recurrent disease with an expected survival of less than two years[1]. Patients treated with curative intent are usually included in some form of

Table 1 Benefits and side effects of colorectal cancer surveillance

Benefits	Harms
Reassurance of surveillance	Impact of false positive tests
For the CRC survivor	Over diagnoses
For spouses and family	Complications related to the screening tests
Improved survival	Labeled as sick or at high risk
Control of treatment effects	False assurance of disease free status
Is the societal harm-to benefit ratio acceptable?	

CRC: Colorectal cancer.

preventive follow-up program involving periodic evaluations. Reviews comparing various follow-up programs have suggested that more intensive follow-up strategies tend to increase the five-year survival rate by 5%-10%[2,3].

Most national follow-up programs recommend intensive follow-up. However, there exist controversies on how to define an "intensive" follow-up program. This is mirrored in the fact that two identical national follow-up programs do not exist. In general, an intensive follow-up program consists of regular testing (usually every 3 mo the first two years) and consultations, whereas a low intensive follow up program is defined as no regular testing and consultations. In addition, most national follow-up programs make a distinction between rectal cancer and colon cancer surveillance, which is reflected in the difference of recommended radiological test modalities.

However, all preventive programs have the potential to harm patients[4-6]. The potential survival benefits of a follow-up program for CRC cancer patients have been well described, but much less is known about the potential negative effects accruing to patients and their families[2,3]. Patients surgically treated for CRC have to decide in partnership with the treating surgeon or family physician, whether they should participate in a CRC follow-up program. In making this personal decision, it is important to know not only the magnitude of potential benefits, but also the magnitude and likelihood of the potential adverse and unintended effects[5].

Firstly the survival benefits of intensive CRC follow-up must be delineated. In general, the benefits of preventive programs can be described as: (1) relative reduction of mortality rate; (2) absolute reduction of mortality; (3) the number of patients needed to prevent one adverse event; (4) evaluation of treatment effect; (5) reassurance by follow-up leading to improved quality of life (QoL); and (6) detection of other diseases[4]. In this paper we will further elaborate these terms.

Secondly, the side effects of CRC follow-up must be compared to the survival benefits. Well-known side effects of preventive programs are (1) over-diagnosis; (2) somatic complications caused by testing; (3) negative psychological consequences of follow-up; and (4) impact of a false positive (leading the patient to believe that he or she has recurrent disease) or false negative (leading to a potential diagnostic delay) tests.

Thirdly, the net benefits of follow-up must be considered in light of the associated economic costs. The United Kingdom's National Institute for Health and Clinical Excellence (http://www.nice.org.uk) has proposed a societal willingness-to-pay of £40000 per life year gained, but this upper limit is controversial. In the case of CRC follow-up, it means that the long-term benefits of a follow-up program (*i.e.,* the attempted curative resection of recurrent disease and resulting gains in survival) have to be balanced against society's willingness to pay for such a service. To our knowledge, a systematic comparison of the benefits *vs* side effects of CRC follow-up has not been performed. Thus, the objective of this paper is to summarize the existing evidence regarding the benefits and side effects of CRC follow-up. An overview of the potential benefits and harms of CRC follow-up is provided in Table 1.

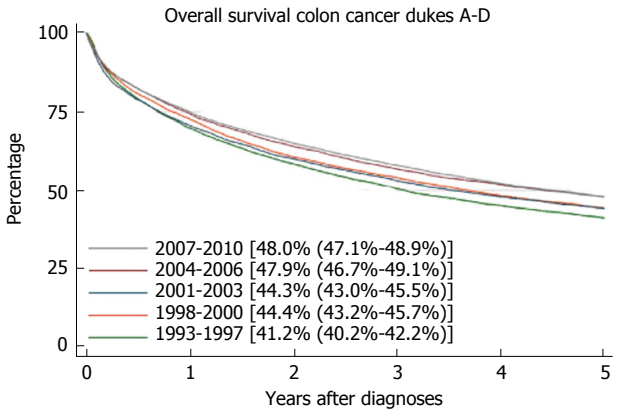

Figure 1 Overall survival of colon cancer dukes A-D. Eighty percent of the recurrences occur within the 3 first years after initial treatment, which is used as an argument to perform intensive surveillance the first 3 years. After 5 years, the survival curve is steady with few deaths caused by colon cancer. Courtesy of the Norwegian Cancer Registry (http://www.kreftregisteret.no/en/).

RESEARCH

We performed a systematic PubMed search with the medical subject heading (MeSH) keywords "colorectal" in combination with the keywords "follow-up", "surveillance", "cancer recurrence"; "risk benefit assessment" and "false positive reactions". Inclusion of papers was decided by discussion among authors. All reference lists of included publications were searched for relevant publications. Finally we identified relevant publications from the author's personal databases. This resulted in 60 publications included in the review.

Benefits of colorectal follow-up

Benefit: Improved survival: The recurrence rate in CRC has been reported to be 30%-40% within 5 years (Figure 1)[1]. This means that all follow-up programs must focus on the early detection of recurrent cancers, aiming to offer curative metastases surgery to as many patients as possible.

Two contemporary meta-analyses revealed that intensive and less intensive follow-up led to detection of

a similar number of recurrences but that detection occurred between 5.91 mo (95%CI: 3.09-8.74) and 6.75 mo (95%CI: 2.44-11.06) earlier with intensive follow-up. Both analyses also found that curative reoperation for metastasis was significantly more likely in those subjects who were followed up intensively (Tjandra *et al*[2]: OR = 2.41, 95%CI: 1.63, 3.54. Jeffery *et al*[3]: OR = 2.81, 95%CI: 1.65-4.79). The survival benefits of intensive CRC follow-up has been reported to be a 5%-10% reduction in the total cohort mortality rate. The increased overall survival, earlier detection of recurrence, and higher reoperation rates observed provide only circumstantial evidence that intensive follow-up extends life by making cure of recurrent disease more likely. Neither meta-analyses found that cancer specific survival was improved by intensive follow-up.

However, there exists limited data regarding the relative reduction in mortality or number of patients who must be followed intensively in order to save one life from recurrent cancer death. Factors other than intensive follow-up have been postulated to contribute to the mortality reduction associated with CRC follow-up. Some combination of increased psychological well-being, improved health behavior, and improved treatment of coincidental disease may contribute to the mortality benefit. This issue represents an important direction for future studies[7].

Recently, the results from the follow-up after colorectal surgery (FACS) trial were reported[8,9]. The factorial randomized trial design, with independent allocation to the carcinoembryonic antigen (CEA) and computed tomography (CT) interventions, meant that patients received 1 of 4 types of follow-up: (1) CEA follow-up: measurement of blood CEA every 3 mo for 2 years, then every 6 mo for 3 years, with a single chest, abdomen, and pelvis CT scan at 12 to 18 mo if requested at study entry by hospital clinician; (2) CT follow-up: CT of the chest, abdomen, and pelvis every 6 mo for 2 years, then annually for 3 years; (3) CEA and CT follow-up: both blood CEA measurement and CT imaging as above; and (4) Minimum follow-up: no scheduled follow-up except a single CT scan of the chest, abdomen, and pelvis at 12 to 18 mo if requested at study entry by the hospital clinician.

Interestingly, there were no differences seen in overall or cancer-specific mortality between any of the intensive arms and the minimum follow-up group. Most patients with recurrence suffered from incurable disease. In fact, only 71 (5.9%) of 1202 patients followed were suitable for potentially curative treatment. Significantly more patients were treated with curative intent in the intensive follow up groups compared to minimalist follow-up, but there were no difference in the number of total deaths in the two groups. These data argue against very intensive follow-up schedules.

In conclusion, although two meta-analyses have reported a 5%-10% reduction in overall mortality among patients undergoing intensive follow-up, the existing evidence of any benefit in terms of cancer-specific survival is limited. The results from the FACS trial did not show any compelling evidence of a significant survival benefit of CRC follow-up. Hopefully, the final results of the ongoing COLOFOL trial will help answer the debate regarding which follow program enables the highest survival[10]. A summary of randomized controlled trails and their potential survival benefit is provided in Table 2.

Benefit: Control of treatment effects: There exist several international controversies around treatment (drains *vs* no drains, laparoscopic technique *vs* open technique among others) and follow-up of patients with CRC[11,12]. There are for instance no similarly designed follow-up program at an international level[13-16]. It is therefore imperative for improved CRC treatment quality that the effects of radio-chemotherapy, surgical technique and postoperative follow-up are continuously evaluated, and a structured follow-up program might be a way to perform such a quality control[17,18].

Benefit: Reassurance of follow-up: There is no existing evidence that participation in a follow-up program leads to increased personal well-being. Some researchers have investigated the psychological effects of CRC follow-up[19-22]. None of the resulting studies have found improvement in the patient QoL with follow-up.

Harms of CRC follow-up

Harm: False positive tests: Table 3 summarizes the false positive rates of the most commonly used CRC follow-up tests. As an illustration, consider a patient followed according to the most recent United States follow-up recommendations from the National Comprehensive Cancer Network[16]. Based on the most optimistic estimates in Table 3 the annual probability of at least one false positive test for a patient with no actual recurrence would be 41% in each of years one and two, and 28% in each of years three, four, and five. Over the entire five-year period, the probability of at least one false positive would be 87%.

Given their high likelihood, it is important to consider the possible consequences of false positive follow-up tests. Primarily, these can come in the form of economic costs and psychological impact. None of the prospective studies or economic models focusing on CRC recurrence have reported the economic costs of false positive follow-up tests, but quantifying these costs could provide important perspective.

While no studies appear to have specifically addressed the psychological or quality-of-life impact of false positive follow-up tests in colorectal or other types of cancer, a small number of investigators have examined the quality-of-life impact of false positive cancer screening tests. In general, these studies have shown increased anxiety following false positive screening results for as long as 18[23] to 24[24] mo after the false positive result[23,25,26]. This data comes from populations who have not previously

Table 2 Comparison of randomized trials assessing follow-up after colorectal cancer curative surgery *n* (%)

Trial	Cancer stage included	Enrolled (*n*)	Recurrences	Time to cancer detection (mo)	Metastases surgeries (*n*)	Overall 5-yr survival	Disease free 5-yr survival	Survival after met surgery
Ohlsson 1995								
Total	Dukes A, B, C	107	35 (33)					
Intensive		53	17 (32)	20 (median)	5	75	78	29% 5 yr
Control		54	18 (33)	24 (median)	3	67	71	22% 5 yr
Makela 1995								
Total	Dukes A, B, C	106	43 (41)		8	58		Overall 3 pts mean 26 mo survival
Intensive		52	22 (42)	10 (mean)	5	59		
Control		54	21 (39)	15 (mean)	3	54		
Pietra 1998								
Total	Dukes B, C	207	82 (39)					Overall 8 pts mean 29 mo survival
Intensive		104	41 (39)	10.3 (mean)	21	73	68	
Control		103	41 (40)	20.2 (mean)	6	58	53	
Rodriegez-Moranta 2006								
Total	TNM Ⅱ and Ⅲ	259	69 (26)					NA
Intensive		127	35 (27)	39 (mean)	18	75	NA	
Control		132	34 (26)	38 (mean)	10	73		
Secco 2001								
Risk adapted intensive	Low risk *vs* high risk	108	74 (68)	Total	31	48	NA	NA
Risk adapted low intensive		84	27 (32)	13.5 (mean)		82		
Minimal follow-up: High risk		84	58 (69)		13	35		
Minimal follow-up: Low risk		61	25 (40)			60		

NA: Not available.

Table 3 Probability of false positive test results (1-specificity) for commonly used colorectal cancer follow-up tests

Test	False positive rate (1-specificity)	Ref.
Serum CEA	10%	[54]
CT-hepatic metastases	5%-28%[1]	[55-58]
CT-other abdominal metastases	2%	[58]
Contrast enhanced ultrasound-liver	4%-33%[2]	[56,57,59]
Ultrasound-liver	50%	[59]
CT-lungs	4%	[58]
Colonoscopy	0%	[32]

[1]Based on specificity estimates from individual studies of 89%[55] (*n* = 24), 95%[58] (*n* = 115), 72%[56] (*n* = 87), and 91%[57] (*n* = 100); [2]Based on specificity estimates from individual studies of 96%[60] (*n* = 68), 96%[57] (*n* = 99), and 67%[59] (*n* = 56) subjects. The last was the only to employ intraoperative confirmation of hepatic metastases. The annual probability of at least one false positive test for a patient with no actual recurrence would be 41% in each of year one and two, and 28% in each of year three, four, and five. Over the entire five-year period, the probability of at least one false positive would be 87%. CT: Computed tomography; CEA: Carcinoembryonic antigen.

been diagnosed with and treated for cancer, so the results are difficult to extrapolate to CRC survivors.

Harm: Somatic complications caused by tests: Aside from any unlikely negative sequelae of CT radiation exposure, colonoscopy related colonic perforation and post-procedure bleeding represent the most likely serious complications arising from CRC follow-up. Endoscopic follow-up is endorsed in most comprehensive follow-up recommendations[16,27-31] primarily as a means to detect metachronous CRC's (normally representing between 1.6% and 7.4% of CRC recurrences) or adenomas with advanced features[2,32,33]. The relatively invasive procedure has sensitivity of 95% and specificity of 100% for detecting high-risk polyps or tumours, however the major complication rate has been reported as 0.2%-1.2%[34-36].

To date, no trial has reported increased survival associated with colonoscopy follow-up after CRC resection. Because of the unproven benefit and non-trivial risk, some have argued against routine endoscopic follow-up after curative CRC resection[37-39]. Further study is needed to explore whether CT Colonography may eventually provide a better balance of risks and benefits[38].

Harm: QoL implications: There is limited evidence showing that enrolment in a follow-up program improves QoL among CRC survivors. In fact, available data from breast follow-up trails could be used to support the opposite viewpoint: such follow-up programs and tests might negatively influence QoL[40-42]. It is often claimed-and some evidence corroborates[22]-that follow-up tests can be reassuring for patients, and this may be true if all of the tests are completely normal every time. However, equivocal test results such as a slightly elevated CEA level, or questionable shadows on CT are quite common, and they commonly spur additional testing. This period between initial suggestive test result and subsequent conclusive work-up can be a stressful one for patients[21].

Some researchers have investigated the psychological effects of CRC follow-up[19-22]. None of the resulting studies have found improvement in the patient QoL with

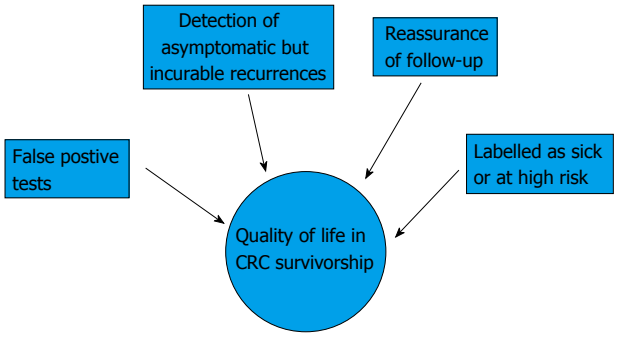

Figure 2 Factors influencing quality of life among colorectal cancer survivors enrolled in a follow-up program. CRC: Colorectal cancer.

follow-up. In a recent published randomized trial comparing general practitioner *vs* surgeon-organized follow-up, there were no differences between the two groups in QoL measured by ERTOC-QLQ C30 and EQ-5D[21]. In fact, both groups had similar QoL levels as the general United Kingdom population at baseline (1 mo postoperatively). Results from a similar 2006 trial by Wattchow *et al*[19] told a similar story. There, study patients remained in the normal range for depression and anxiety with no difference between the two groups at either 12 or 24 mo[19,20]. In recent meta-analyses, it has been shown that anxiety rather than depression was a major problem among long-term cancer survivors. It is however unknown what impact an organized cancer follow-up program has on anxiety[43]. It has been shown that 46 percent of patients reported physiological distress while awaiting the results of a potential cancer diagnosis[44]. This and other trials suggest that tests recommended by a cancer screening or preventive program cause harm in terms of physiological distress[44-46].

The only survey showing a slight improvement in QoL among CRC survivors with intensive follow-up was published in 1997[47]. This survey included 350 Danish participants who reported a small but significant increase in QoL associated with more frequent follow-up, as measured by the Nottingham Health Profile.

In conclusion, there exists very limited evidence that CRC follow-up improves QoL among CRC survivors. Further research is needed, in particular, to address the impact of a false positive follow-up test on QoL among CRC survivors. From breast cancer follow-up trials, there is compelling evidence that postoperative follow-up does not improve QoL and that follow-up testing might cause physiological distress[48]. Factors that may impact QoL in a positive or negative way among colon cancer survivors enrolled in a follow-up program are shown in Figure 2.

DIRECTION OF FUTURE RESEARCH

According to the World Health Organisation, the success of preventive programs depends on three fundamental principles (www.who.int/cancer/detection/variouscancer/en/): The target disease should be a common form of cancer, with high associated morbidity or mortality; Effective treatment, capable of reducing morbidity and mortality, should be available; Test procedures should be acceptable, safe, and relatively inexpensive.

In CRC follow-up these principles are fulfilled: (1) CRC is the third most common cancer disease, and the risk of recurrence is as high as 30 to 40 percent; (2) if successful, metastasectomy can be curative (*i.e.,* R0 resections); and (3) the tests in most programs are acceptable, relatively safe and relatively inexpensive. However, as discussed, there are several potential side effects of CRC follow-up; future research much be directed at further exploring these harms and weighing them against the expected survival benefit. Recently, a survey published in British Medical Journal found that the harms of screening and preventive programs were poorly reported[49]. Healthcare decision makers, surgeons, and patients therefore cannot make informed choices.

Personalized medicine is defined as a medical model that proposes the customization of healthcare, with medical decisions, practices and tests being tailored to the individual patient. To our knowledge there exist no individual risk stratification in the different national colorectal follow-up guidelines, and this is an area of future research.

Firstly we believe that genetic testing and biological determinants of tumor recurrence will gain increasingly importance[50,51]. The individualization of cancer care requires a deep understanding of tumor biology and the identification of tumor subsets that offer targets for tumor specific treatment. Of specific interest for CRC follow-up programs, are the promising results of the 12-gene recurrence score (RS), which is a quantitative assay integrating stromal response and cell cycle gene expression. It is shown that the 12-gene RS predicts recurrence in stage Ⅱ colon cancer. This tool appears promising as a means to inform decision making around adjuvant chemotherapy following resection of stage Ⅱ colon cancer. The use of the tool in planning post-treatment follow-up does not appear to have been explored, however[52].

Secondly, test intensity, test modality and the risk of false positive events has to be discussed in details with the patient. As shown in Table 3, the probability of at least one false positive event during a five-year follow-up program might be as high as 87%. High-test intensity programs should be offered to patients with a high probability of recurrent cancers, but this must be weighed against the patient's preferences of experiencing a false positive test.

Finally, research must be aimed to identify the optimal combination of test, blood samples and clinical examinations that creates the highest possible overall follow-up sensitivity and specificity.

CONCLUSION

Any survival benefit (or lack of benefit) of the CRC fol-

low-up must be considered along with the views of the patients to ensure that follow-up programs are accessible and acceptable, and that they address all patient needs and concerns. However, the problem of postoperative cancer follow-up is that a vast majority of patients must undergo a large number of tests without any benefit, or even with some harm, to identify a small number of patients with curable recurrence. Patients with asymptomatic but incurable disease (10%-20% of all recurrences) likely represent the group with the most potential to be harmed by follow-up[21,53].

In conclusion, little is known about the potential harms of CRC follow-up, especially when it comes to the impact of false positive tests. Tailored follow-up programs based on the individual's risk of cancer recurrence and likely metastatic spread pattern must be developed. Further research is needed to settle these controversies, and new methods of decision-analytic modeling in combination with the emerging data from COLOFOL must be applied[9,10].

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REVIEW

Esophageal cancer: A Review of epidemiology, pathogenesis, staging workup and treatment modalities

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Abstract

Esophageal cancer is a serious malignancy with regards to mortality and prognosis. It is a growing health concern that is expected to increase in incidence over the next 10 years. Squamous cell carcinoma is the most common histological type of esophageal cancer worldwide, with a higher incidence in developing nations. With the increased prevalence of gastroesophageal reflux disease and obesity in developed nations, the incidence of esophageal adenocarcinoma has dramatically increased in the past 40 years. Esophageal cancer is staged according to the widely accepted TNM system. Staging plays an integral part in guiding stage specific treatment protocols and has a great impact on overall survival. Common imaging modalities used in staging include computed tomography, endoscopic ultrasound and positron emission tomography scans. Current treatment options include multimodality therapy mainstays of current treatment include surgery, radiation and chemotherapy. Tumor markers of esophageal cancer are an advancing area of research that could potentially lead to earlier diagnosis as well as playing a part in assessing tumor response to therapy.

Key words: Esophageal cancer; Esophageal cancer staging; Esophageal squamous cell carcinoma; Esophageal adenocarcinoma; Surgery

Core tip: Esophageal carcinoma is a serious malignancy with regards to mortality and prognosis, and is expected to increase in incidence over the next 10 years. Squamous cell carcinoma is the most common histological type of esophageal cancer worldwide but the incidence of esophageal adenocarcinoma has dramatically increased in the past 40 years. Esophageal cancer is staged according to the TNM system. Common imaging modalities used in staging include computed tomography, endoscopic ultrasound and positron emission tomography scans. Current treatment options include multimodality therapy. Including surgery, radiation and chemotherapy. Tumor markers of esophageal cancer are an advancing area of research that could potentially lead to earlier diagnosis.

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INTRODUCTION

Esophageal cancer is considered a serious malignancy with respect to prognosis and mortality rate. Account-

ing for more than 400000 deaths worldwide in 2005[1]. Esophageal carcinoma is the eighth most common cancer, and the sixth most common cause of cancer related deaths worldwide with developing nations making up more than 80% of total cases and deaths[2]. Over 490000 new cases of esophageal cancer were reported in 2005. While many other types of cancer are expected to decrease in incidence over the next 10 years by 2025 the prevalence of esophageal cancer is expected to increase by 140%[1]. According to the National Cancer Institute, in the United States there will be approximately 17990 new cases and 15210 deaths in 2013[3]. Despite many advances in diagnosis and treatment, the 5-year survival rate for all patients diagnosed with esophageal cancer ranges from 15% to 20%[4]. The epidemiology of esophageal cancer in developed nations has dramatically changed over the past forty years. Forty years ago squamous cell carcinoma (SCC) was responsible for greater than 90% of the cases of esophageal carcinoma in the United States. Adenocarcinoma has now become the leading cause of esophageal cancer in the United States, representing 80% of cases[5]. In 1975 esophageal adenocarcinoma (EAC) affected four people per million, in 2001 the rate had increased to twenty-three people per million. Making it the fastest-growing cancer in United States, according to the National Cancer Institute[6]. Considerable differences of incidence of esophageal cancer exist on the basis of geographic and racial differences, which can be linked to differences in exposure to risk factors. This review discusses epidemiology, pathogenesis, etiology and treatment modalities available for esophageal cancer.

EPIDEMIOLOGY

Worldwide SCC is the most prevalent histological type of esophageal cancer, while in certain developed nations including Australia, Finland, France, United States and United Kingdom adenocarcinoma of the esophagus predominates[7]. Esophageal cancer incidence and histological type is highly variable based upon geographic location. Incidence rates of SCC of the esophagus have been reported as high as 100 cases per 100000 annually in an area referred to as the "Asian esophageal cancer belt" and this region extends from northeast China to the Middle East[8]. In the United States the National Cancer Institute estimates close to 18000 new cases and more than 15000 deaths from esophageal cancer in 2013[3]. From 1975 to 2004, the incidence of EAC among white American males increased by more than 460% and in the same period, the incidence among white American females increased by 335%[9].

PATHOGENESIS

The two most common histological types of esophageal carcinoma include SCC and adenocarcinoma. Less than 1% to 2% of all esophageal cancers are sarcomas or small cell carcinomas[10]. Rarely lymphomas, carcinoids, and melanomas may arise in the esophagus.

PATHOGENESIS OF SCC

SCC is the most common type of esophageal cancer worldwide. The overall incidence increases with age, reaching a peak in the seventh decade. SCC occurs equally as often in the middle and lower esophagus, with an incidence that is three times higher in blacks in comparison to whites[11].

Major risk factors include alcohol consumption and tobacco use. Most studies have shown that alcohol is the primary risk factor but smoking in combination with alcohol consumption may have a synergistic effect and increase the relative risk. The relative risk in men who used both heavy tobacco and alcohol was 35.4 in white males and 149.2 in black males compared to men of the same race and region who were non-smokers or drinkers[12]. The mechanism of how tobacco and alcohol in combination lead to increased risk of esophageal cancer has been extensively studied. Alcohol can damage the cellular DNA by decreasing metabolic activity within the cell and therefore reduce detoxification function while promoting oxidation[13]. Alcohol is a solvent, specifically of fat-soluble compounds. Therefore, the hazardous carcinogens within tobacco are able to penetrate the esophageal epithelium easier[14]. Some of the carcinogens in tobacco include aromatic amines, nitrosamines, polycyclic aromatic hydrocarbons, aldehydes and phenols.

Other carcinogens, such as nitrosamines found in certain salted vegetables and preserved fish, have also been implicated in SCC of the esophagus. The pathogenesis appears to be linked to inflammation of the squamous epithelium that leads to dysplasia and in situ malignant change[15].

PATHOGENESIS OF ADENOCARCINOMA

Adenocarcinoma of the esophagus occurs in the distal esophagus approximately three-fourths of the time[16] and has a distinct link to gastroesophageal reflux disease (GERD). Untreated GERD can progress to Barrett's esophagus (BE), where the stratified squamous epithelium that normally lines the esophagus is replaced by a columnar epithelium. The chronic reflux of gastric acid and bile at the gastroesophageal junction and the subsequent damage to the esophagus has been implicated in the pathogenesis of Barrett metaplasia[17]. The exact nature of the metaplasia still remains to be determined. Diagnosis of Barrett esophagus can be confirmed by biopsies of the columnar mucosa during an upper endoscopy. According to the requirements set forth by the United States gastroenterology societies, the biopsy specimen should contain the characteristic columnar epithelium metaplasia with goblet cells for a definitive diagnosis. Barrett esophagus incidence increases with age and is uncommon in children. It is more common in men than women and more common in whites in comparison to Asian or African American populations.

Some studies have shown that the risk of adenocarcinoma of the esophagus may be affected by the extent of esophagus lined by esophageal metaplasia[18]. The longer the segment of esophagus affected the higher the risk of adenocarcinoma. However, given the fact that short segment esophageal metaplasia is more common in the general population, many cases of adenocarcinoma occur in patients with short-segment metaplasia. Less than five percent of patients diagnosed with adenocarcinoma of the esophagus had a prior diagnosis of BE[19]. The risk of developing esophageal cancer is 50-100 times more likely in those patients with BE[15]. However, a majority of patients with BE will not develop EAC, the annual risk in patients with BE has been reported as 0.12%[20].

Screening for BE *via* endoscopy is controversial and challenging. Currently no definitive screening protocol has been formulated due to lack of documentation that screening effects EAC mortality. A large number of patients with BE will not have reflux symptoms therefore predicting which patients will have BE prior to endoscopy is very challenging. Despite no definitive data for universal recommendation, most gastroenterological associations consider endoscopic surveillance "reasonable" and "desirable" in patients with diagnosed BE[21]. The primary goal of surveillance is to identify dysplasia before it progresses to an invasive malignancy. Current endoscopic technique consists of four quadrant biopsies taken every 2 cm in the columnar-lined esophagus for histological evaluation. The American College of Gastroenterology has recommendation guidelines for how often surveillance should take place based upon the presence or absence of dysplasia and grade of dysplasia if present. Surveillance endoscopy is recommended every 2-3 years in patients with no dysplasia. In patients with low-grade dysplasia, surveillance is recommended every 6 mo for the first year. If the dysplasia has not progressed in the first year, yearly surveillance is applicable. In patients diagnosed with high-grade dysplasia (HGD), two alternatives have been proposed. One option is to continue intensive endoscopic surveillance every 3 mo until intramucosal cancer is detected. The other alternative is for the patient with HGD to undergo endoscopic mucosal resection[20]. Although the natural history of HGD is variable, > 30% of patients with HGD will develop EAC within 5 years[22]. Due to the high risk of cancer most patients with HGD are evaluated as if cancer is present.

Another risk factor for EAC is obesity, specifically in those individuals with predominately abdominal centered fat distribution. Hypertrophied adipocytes and inflammatory cells within fat deposits create an environment of low-grade inflammation and promote tumor development through the release of adipokines and cytokines[23]. Adipocytes in the tumor microenvironment supply energy production and support tumor growth and progression[22].

Long-term prognosis after resection is better for adenocarcinoma compared to SCC. A study by Siewert *et al*[24]. Of 1059 patients who underwent resection showed the overall 5-year survival rate for the adenocarcinoma group was 47% in comparison to 37% for the group with SCC.

ROUTES OF ESOPHAGEAL CANCER SPREAD

Prognosis in esophageal cancer is greatly dependent on local invasion as well as spread to regional and distant structures within the body. Esophageal cancer is notoriously aggressive in nature, spreading by a variety of pathways including direct extension, lymphatic spread and hematogenous metastasis. The lack of serosa in the esophageal wall plays an integral role in the local extension of esophageal cancer. With no anatomical barrier, the primary tumor is able to extend rapidly into the adjacent structures of the neck and thorax including the thyroid gland, trachea, larynx, lung, pericardium, aorta and diaphragm[25]. The lymphatic drainage of the esophagus is extensive. It is drained by two separate lymphatic plexuses, with one lymphatic plexus arising within the mucosal layer and a second plexus arising within the muscular layer. A majority of the lymphatic fluid from the upper two-thirds of the esophagus tends to flow upward, and the lymph from the lower third of the esophagus flows relatively downward, but all the lymphatic channels of the esophagus communicate. Therefore, lymphatic fluid from any portion of the esophagus may spread in either direction and spread to the intrathorax or intra-abdomenal lymph nodes[26]. Esophageal cancer also spreads hematogenously,, in order of decreasing frequency, to the liver, lungs, bones, adrenal glands, kidney and brain. This method of spread is more common with more advanced stages of esophageal cancer[27].

STAGING OF ESOPHAGEAL CANCER

The clinical staging of esophageal cancer is assessed with the widely accepted TNM system developed by the American Joint Committee on Cancer (AJCC). Pretreatment staging of esophageal cancer will directly affect overall treatment options available to each patient and their prognosis, so accurate staging is essential.

T staging of esophageal cancer focuses on identifying the depth of invasion of the primary tumor. A critical aspect of T staging focuses on establishing if the primary tumor has invaded the surrounding mediastinal structures, given that these patients would no longer be considered surgical candidates. Table 1 describes the TNM system, specifically referring to depth of invasion in T staging[28]. This aspect of staging is essential in determining stage-specific protocols for treatment (Table 2[28]). For example, for T3 or T4 tumors the oncology team will use preoperative chemotherapy or combination radiation and chemotherapy in order to render the primary tumor resectable by surgical excision. In contrast, T1 or T2 tumors are treated primarily with surgical resection[29]. Given the importance of T Staging in treatment options

Table 1 TNM system, specifically referring to depth of invasion in T staging

Category	Description
Tis	Carcinoma *in situ*
T1	Tumors invade lamina propria or submucosa
T2	Tumors invade muscularis propria
T3	Tumors invade adventitia
T4	Tumors invade adjacent structures
N0	No regional lymph node metastases
N1	Regional lymph node metastases
M0	No distant metastasis
M1a, M1b	Distant metastasis

and overall prognosis, many modalities have been utilized to accurately establish T Stage. These options include computer tomography (CT), endoscopic ultrasound (EUS) and ^{18}F-fluorodeoxyglucose positron emission tomography (FDG-PET scan)[30].

T STAGE OF ESOPHAGEAL CANCER

When assessing the esophagus by CT, a basic starting point to consider is the esophageal wall thickness. A wall thickness greater than 5mm is considered abnormally thick[31] given that the distended wall of the esophagus is usually less than 3 mm[32]. Esophageal wall thickness asymmetry is a classic but nonspecific CT finding of esophageal cancer and esophageal wall thickness symmetry should always be considered when evaluating the esophagus by CT. CT has been shown to be less accurate when compared to other assessment modalities such as EUS[33]. CT assessment of the esophagus is also unable to accurately differentiate between T1, T2 and T3 stages of the primary tumor invasion. This information is essential in order to guide stage-specific protocols of treatment. The most useful aspect of CT imaging in determination of T status is evaluating if the primary tumor invades into adjacent structures. Obliteration of the fat planes between the primary tumor and the adjacent structures on CT would establish the primary tumor as a T4 stage cancer. The sensitivity and specificity of CT to detect mediastinal invasion ranges between 85%-100%[34,35]. It should be noted that while obliteration of the fat planes between the primary esophageal tumor and adjacent structures is usually reliable in the establishment of a T4 stage tumor, it can occur in patients with prior radiation therapy or cachectic patients.

EUS is now considered the most accurate imagining modality available to establish T staging of esophageal cancer. In comparison to CT, EUS is more accurate to differentiate between T1, T2 and T3 tumors[36]. In comparing the two imaging modalities, EUS was able to determine the preoperative T stage 76%-89% in comparison to 49%-59% when CT imaging was utilized[37-39]. This differentiation is essential in guiding stage-specific treatment protocols and the overall prognosis. Overall in a study conducted by Rösch[40], EUS was able to correctly stage esophageal cancer 84% of the time, and the accuracy improved as the T stage of the primary tumor increased. Accuracy ranged from 75%-82% for the T1 disease state to 88%-100% for the T4 disease state[41]. EUS is a useful tool in assessing the extent of disease as well as response to chemotherapy, when the dimensions of the tumor are analyzed as the primary variable. However, EUS is unreliable for staging esophageal cancer after neoadjuvant chemoradiation[42]. Other potential limitations of EUS do exist. With any form of ultrasound the accuracy of the study is operator dependent. Also, in cases of esophageal cancer where the esophageal lumen has been narrowed by strictures or stenosis, it may not be possible to pass the endoscope through to visualize the entire tumor[30].

N STAGE OF ESOPHAGEAL CANCER

In esophageal cancer, N Staging can be defined by the involvement (N1) or absence of involvement (N0) of periesophageal lymph nodes. Sensitivity and specificity of CT scans to detect periesophageal lymph node involvement depends on the size of the lymph nodes. Most studies, used the common size criteria of 1 cm to define a lymph node as enlarged. Sensitivity was reported as 30%-60% while specificity was 60%-80%[43]. An obvious limitation of CT imaging in the ability to detect nodal involvement, comes from the possibility that a normal sized lymph node may contain metastatic foci without an obvious increase in the size of the lymph node. Also, an enlarged lymph node does not necessarily mean metastasis, given that benign enlargement and inflammation may occur[43]. Accuracy to detect N stage by CT imaging was reported as 46%-58%[39].

EUS has been shown to be more accurate in determining nodal involvement in esophageal cancer, with an accuracy of 72%-80%[44]. Accuracy has increased greatly with the use of EUS in combination with United States guided fine-needle aspiration to evaluate for lymph node metastasis.

FDG-PET has also been utilized in determining nodal involvement in esophageal cancer. Assessment of local and regional lymph nodes for uptake of FDG is difficult to determine given the intense uptake of FDG by the primary esophageal tumor. However, PET is quite useful in detecting distant metastasis, including metastasis to the abdomen and cervical lymph nodes. Sensitivities were reported as high as 90% in distant lymph node metastasis[45].

M STAGE OF ESOPHAGEAL CANCER

Esophageal cancer is notoriously aggressive and invasive in nature. In fact 20%-30% of patients with esophageal cancer will have distant metastasis at time of initial diagnosis[27]. The presence or absence of distant metastasis will be essential in guiding treatment options and in determining operability. Common sites of distant metastasis include liver, lung and bones[30].

Table 2 Aspect of staging is essential in determining stage-specific protocols for treatment

Stage	Tumor	Node	Metastasis	Therapeutic options
0	Tis	N0	M0	Local ablative therapy
I	T1	N0	M0	Surgery
ⅡA	T2	N0	M0	Surgery
	T3	N0	M0	
ⅡB	T1	N1	M0	Neoadjuvant therapy with or without surgery
	T2	N1	M0	
Ⅲ	T3	N1	M0	Neoadjuvant therapy with or without surgery
	T4	Any N	M0	
ⅣA	Any T	Any N	M1a	Chemotherapy or radiation therapy with or without surgery
ⅣB	Any T	Any N	M1b	Palliative treatment

In the classification system of metastasis set forth by the AJCC, distant metastasis can be subdivided into M1a and M1b. Each of these classifications is crucial in determining possible treatment options. M1a includes metastasis to celiac and cervical lymph node groups. This classification is associated with a better prognosis compared to M1b. Patients classified as M1a often times complete a course of neoadjuvant therapy followed by surgical resection. Patients with M1b include those with distant site metastasis. This classification usually carries a worse prognosis given that surgical resection with curative intent is not indicated in these cases[46].

CT is the most commonly used imaging modality to rule out distant metastasis in patients with esophageal cancer. The most common areas of distant metastasis can be quickly assessed using contrast-enhanced CT. Sensitivity for spiral CT to detect masses ⩾ 1 cm has been reported as high as 90%[47].

EUS is limited in its ability to assess for distant metastasis. In general, CT or FDG-PET is preferred over endoscopic United States for M staging of esophageal cancer.

FDG PET most distinct role in esophageal cancer staging is in the detection of distant metastasis. In comparison to CT, PET has been shown to be more accurate in detecting distant metastasis[48]. One study showed that PET was able to detect distant metastasis 15% of the time in patients that were believed to only have primary esophageal cancer by other imaging modalities[49]. If present, distant metastasis places the patient in M1b category and surgery with curative intent is no longer recommended. Accurate M staging is imperative in guiding treatment options.

TUMOR MARKERS

Serum human relaxin 2 (H2 RLN) is made in the corpus luteum of females and the prostate of males. It helps remodel various tissue components such as extracellular matrix, collagen, and matrix metalloproteinase. There is supporting evidence that RLN is a tumor growth factor and has been shown *in vitro* to enhance invasiveness of breast cancer cells. A study measuring RLN levels in patients with esophageal SCC (ESCC) discovered that patients with higher levels of H2 RLN had more distant metastasis, lymph node metastasis, higher clinical stage, and a shorter survival rate. This study demonstrated the possibility of using H2 RLN as a serum prognostic factor for ESCC[50]. A Japanese study, investigated the prognostic value of the tumor marker p53 in ESCC. They observed no correlation between a p53 aberration and any clinical, pathological, or epidemiology of ESCC[51]. Another study investigated the marker gene, WDR66 through genome-wide expression profiling. Other WD proteins have been used as tumor markers in other cancers, such as hepatocellular carcinoma. WDR66 has a higher concentration in ESCC tissue than healthy tissue. WDR66 was found to have a role in the growth, motility, and epithelial-mesenchymal transition of ESCC. Poor survival was noted with high levels of WDR66 in the tumor tissue[52]. In a Chinese study, the gene marker phospholipase A2 group ⅡA (PLA2G2A) was investigated to determine its usefulness as a prognostic factor of ESCC. PLA2G2A catalyzes multiple fatty acids, including arachidonic acid and is expressed in colorectal, pancreatic, prostate, gastric and lung cancer. Low expression of PLA2G2A in tumor tissue correlated to high-grade tumors, metastasis, increased depth of invasion, lymphatic invasion, and poorer overall survival rate[53].

PROGNOSTIC FACTORS

Platelet count has been used to help determine the prognosis of other cancers because platelets are an integral component of the inflammation processes. Platelet count is inversely related to the cancer prognosis, as in a higher platelet count correlates to a poorer prognosis. The absolute cut off for platelet count as a prognostic factor has been debated. In one study of ESCC, platelet counts were higher in patients with large tumors. It was determined that those patients with platelet counts ⩽ 205000 had a better 5-year survival rate than patients with platelets > 205000 especially when nodes were involved[54].

Tumor length is used as a prognostic factor in ESCC but the length cutoff point in predicting survival has been contested. Researchers in China looked at tumor length in the elderly population (over 70 years old) and the cutoff point was calculated to be 4.0 cm. Patients with a tumor length of ⩽ 4.0 cm had a better 5-year survival than those with a tumor length of > 4.0 cm, espe-

cially with a T3-4 grade or nodal-negative patients[55].

Cancer causes a hypercoagulable state and this environment encourages tumors to grow and produce more pro-coagulants. D-dimers are the end product of fibrin and fibrinolysis and have been reported to be associated with tumor prognosis, tumor stage, lymph node involvement, and overall survival. One study looked at the plasma D-dimer levels in patients with esophageal cancer before and after surgery as well as patients without cancer. Their research showed that high levels of D-dimers in the pre-operative state correlated with a higher tumor stage and surgery caused more patients to have a hypercoagulable state which shortened their survival time[56].

Nutrition is an important factor that influences patients with esophageal cancer during their perioperative period. Early enteral nutrition was noted to protect the intestinal mucosa, improved the nutritional status, and increased the immune status patients undergoing esophagectomy. Enteral nutrition protected the intestinal mucosa by maintaining the intestinal barrier against plasma endotoxins[57]. Another study looked at immunonutrition in patients with head and neck cancer and esophageal cancer undergoing chemoradiotherapy. Plasma levels of arginine, eicosapentaenoic acid, docosahexaenoic acid, and nucleotides were measured in patients undergoing chemoradiotherapy, who received either an Immune modulating Enteral Nutrition formula (IEN) or an isocaloric, isonitrogenous formula, Standard Enteral Nutrition (SEN). IEN patients had less weight loss, increased antioxidants, and maintained their functional capacities compared to those with the SEN formula[58].

TREATMENT

Surgery can be a definitive treatment for Tis, T1 and some T2 carcinoma of the esophagus. There is some debate on whether neoadjuvant chemoradiotherapy or surgery be performed first on T2 esophageal cancer because staging difficulties[59]. There are different surgical techniques for esophagectomy but the main two are transhiatal esophagectomy (THE) and transthoracic esophagectomy. THE does not include a thoracotomy and instead the stomach is mobilized from the surrounding omentum and blood vessels through a midline supraumbilical incision during the abdominal phase[56]. The esophagus is removed from a small cervical incision usually on the left side of the neck during the cervical phase. The transthoracic esophagectomy uses the Ivor Lewis method, the McKeown Modification (3 hole approach), or the left transthoracic approach. Surgeons choose the method based on tumor location and size. The McKeown modification is performed more for middle and upper esophageal cancer while tumors in the lower third of the esophagus are best approached using the left transthoracic approach[56]. The abdominal phase of the transthoracic esophagectomy is identical to the THE and the thoracic phase is accomplished with a posterolateral thoracotomy in the fifth intercostals space. The McKeown modification also includes a cervical phase where the proximal esophagus can be anastomosed to the stomach conduit[60].

Another critical component of esophagectomy is the lymph node dissection. There is debate about which surgical approach is appropriate based upon access, adequacy of the lymph node retrieval, and the lymph node dissection[54]. Each surgical technique have different lymph node retrieval rates based on the surgical exposure of open, laparoscopic or laparoscopic assisted surgery. Laparoscopic surgery offers less blood loss and more patient comfort but not as many lymph nodes can be retrieved compared to the open approach. Placement of a thorascopic port has been shown to provide more exposure into the chest cavity allowing for a more thorough dissection. One study looked at the difference between open and laparoscopic THE without a thorascopic port and found that while the open procedure yielded more lymph nodes this did not affect the patient's overall prognosis[61].

The differences between transthoracic and THE have been extensively debated. A meta-analysis of 52 studies was performed in 2011 comparing the 5 years survival, postoperative morbidity and mortality between transthoracic and transhiatal esophagectomy. The analysis showed that transhiatal method is associated with reduced operating time, length of stay in hospital, postoperative respiratory complications, and decreased early mortality. The transthoracic method is associated with fewer anastomosis leaks, anastomotic strictures, and vocal cord paralysis. There was no significant difference between transhiatal and transthoracic method in 5-year survival rates[62]. These findings agree with two previous meta-analysis conducted in 1999 and 2001[63-64]. This data suggest that the outcome of the esophagectomy does not depend on the surgical method chosen but more on the surgeon's and hospital's experience in dealing with these complex oncological cases[65].

Another treatment option for high grade dysplasia is esophageal mucosal resection (EMR) or esophageal mucosal dissection. EMR dissects the esophageal submucosa to better evaluate and stage early carcinoma[66]. It has been suggested the EMR be performed on lesions with a diameter $\leqslant$ 2 cm and only occurs in less than one third of the esophageal wall circumference. EMR is used in conjuction with radiofrequency ablation therapy and cryotherapy ablation to eradicate BE[67]. In one trial, EMR with radiofrequency ablation eradicated 90% of dysplasia and metaplasia in patients[68].

One study investigated the hemodynamic changes during surgery between patients who underwent a transthoracic *vs* THE and their post-operative changes. It was found that there was no statistical significance between transthoracic and THE in their intraoperative hemodynamic changes. However more vasopressors were used during surgery in patients with transthoracic esophagectomy due to increased hemodynamic liability[69].

MEDICAL AND RADIOLOGICAL TREATMENT

Chemotherapy and radiotherapy are other critical modali-

ties of treatment along with surgery and are used either in a neoadjuvant or adjuvant setting. A patient will receive neoadjuvant chemoradiotherapy for either a T3 or N1 stage disease. According to the 2013 National Comprehensive Cancer Network guidelines of esophageal cancer, the triple therapy drug regimen include paclitaxel/carboplatin, cisplatin/fluoropyrimidine, and oxaliplatin/fluorouracil. The recommended dose of radiation is 41.4-50.4 Gy[70]. However, one study proposes using chemotherapy alone to treat patients with locally advanced esophageal cancer. Their results showed less toxicities and no difference in their five-year survival rate[71].

An article from Cancer Control found that in the United States, neoadjuvant chemoradiotherapy followed by esophagectomy for resectable esophageal cancer, had a better survival rate than those patients treated with surgery alone[72]. A meta-analysis comparing neoadjuvant chemotherapy with surgery *vs* surgery alone showed a survival increase for those patients who underwent neoadjuvant chemotherapy *vs* surgery alone[73].

A Japanese study found that patients < 60 years of age with a hemoglobin ⩾ 13 g/dL who underwent preoperative chemoradiotherapy, survived longer than those patients who did not undergo treatment. Albumin ⩾ 3.5 g/dL was also associated with prolonged survival[74]. Another study recommends that patients with esophageal cancer who are non-resectable or who refuse surgery can still be treated with definitive chemoradiotherapy due to a 2-year survival rate of 40-55[75]. Another Japanese study found that patients undergoing triple chemotherapy and esophagectomy without the prognostic factors of five or more positive lymph nodes, metastasis to the cervical, mediastinal and abdominal lymph nodes, stage Ⅲ or Ⅳ disease, or intramural metastasis had better recurrence free survival than patients with esophageal cancer and one of the unfavorable prognostic factors[76].

CONCLUSION

Esophageal cancer is a serious malignancy with regards to mortality and prognosis. It is a growing health concern that is expected to increase in incidence over the next 10 years. SCC is the most common histological type of esophageal cancer worldwide, with a higher incidence in developing nations. With the increased prevalence of GERD and obesity in developed nations, the incidence of EAC has dramatically increased in the past 40 years. Esophageal cancer is staged according to the widely accepted TNM system. Staging plays an integral part in guiding stage specific treatment protocols and has a great impact on overall survival. Common imaging modalities used in staging include CT, EUS and PET scans. Current treatment options include multimodality therapy mainstays of current treatment include surgery, radiation and chemotherapy. Tumor markers of esophageal cancer are an advancing area of research that could potentially lead to earlier diagnosis as well as playing a part in assessing tumor response to therapy.

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REVIEW

Neoadjuvant treatment for esophageal squamous cell carcinoma

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Abstract

Squamous cell carcinoma and adenocarcinoma are types of esophageal cancer, one of the most aggressive malignant diseases. Since both histological types present entirely different diseases with different epidemiology, pathogenesis and tumor biology, separate therapeutic strategies should be developed against each type. While surgical resection remains the dominant therapeutic intervention for patients with operable esophageal squamous cell carcinoma (ESCC), alternative strategies are actively sought to reduce the frequency of post-operative local or distant disease recurrence. Such strategies are particularly sought in the preoperative setting. Currently, the optimal management of resectable ESCC differs widely between Western and Asian countries (such as Japan). While Western countries focus on neoadjuvant or definitive chemoradiotherapy, neoadjuvant chemotherapy followed by surgery is the standard treatment in Japan. Importantly, each country and region has established its own therapeutic strategy from the results of local randomized control trials. This review discusses the current knowledge, available data and information regarding neoadjuvant treatment for operable ESCC.

Key words: Esophageal cancer; Squamous cell carcinoma; Neoadjuvant therapy

Core tip: Esophageal squamous cell carcinoma (ESCC) is one of the most aggressive malignant diseases. While surgical resection remains the dominant therapeutic intervention for patients with operable ESCC, alternative strategies are actively sought to reduce the frequency of post-operative local or distant disease recurrence. Such strategies are particularly sought in the preoperative setting. This review discusses the current knowledge, available data and information regarding neoadjuvant treatment for operable ESCC.

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INTRODUCTION

Esophageal cancer is the sixth most common cause of cancer-related deaths and the eighth most commonly diagnosed cancer worldwide[1]. The predominant histological types of esophageal cancer are adenocarcinoma and squamous cell carcinoma[2]. Adenocarcinoma of the distal esophagus predominates in the West, whereas squamous cell carcinoma, which tends to localize in the middle thoracic esophagus, predominates in the East, including Japan. In Western societies, esophageal squamous cell carcinoma (ESCC) is associated with low socioeconomic status, a history of smoking and drinking, liver dysfunction, and pulmonary comorbidities[3]. Since both

histological types present as different diseases in terms of epidemiology, pathogenesis, and tumor biology, therapeutic strategies should be separately developed for each histological type.

Although the prognosis for patients with either type of esophageal cancer is poor, the outlook is worse for ESCC patients than for those with adenocarcinoma, according to some studies[4,5]. However, a Surveillance, Epidemiology, and End-results (SEER) study of 4753 cases archived in a database revealed no difference between the two types[6]. Traditionally, both adenocarcinomas and squamous cell tumors have been treated by surgical resection; however, high frequencies of systemic and local tumor recurrence have urged investigations into multimodality therapies that combine surgery with radiotherapy (RT), chemotherapy (CT), and chemoradiotherapy (CRT). In particular, preoperative therapy has been considered for both tumor types. In Western countries, operable esophageal adenocarcinoma is generally treated by neoadjuvant or definitive CRT. While most researchers agree with this strategy, the optimal therapeutic strategy for ESCC remains controversial. Recently, the Japan Clinical Oncology Group study (JCOG9907) demonstrated that preoperative CT with cisplatin (CDDP) plus 5-fluorouracil (5-FU) followed by surgery improves the overall survival of patients with resectable thoracic ESCC[7]. Since then, preoperative CT followed by radical esophagectomy has been accepted as the standard therapeutic approach to resectable cStage Ⅱ/Ⅲ ESCC. This review discusses the current knowledge, rationale, available data and information regarding neoadjuvant treatment for resectable ESCC.

STRENGTHS AND LIMITATIONS OF SURGICAL RESECTION

Radical esophagectomy with radical lymph node (LN) dissection is the accepted gold standard for therapeutic and staging purposes for ESCC patients. Ando *et al*[8] reported that the survival of Japanese patients undergoing esophagectomy for advanced ESCC improved from 1981 to 1995, largely because of advances in surgical technique and perioperative management. In 2006, the Comprehensive Registry of Esophageal Cancer in Japan reported 1-year, 3-year, and 5-year post-esophagectomy survival rates of 83%, 57%, and 48%, respectively[9]. A German study analyzing whether ESCC could be successfully treated by surgery alone indicated a 5-year survival rate of 30% in primarily resected patients[4]. We of course acknowledge that these results may be influenced by the patient selection bias for surgical procedure.

Western and Eastern counties adopt different surgical approaches; Ivor-Lewis type surgery with two-field LN dissection is preferred in the West, while three-field LN dissection is the treatment of choice in the East, especially in Japan. Three-field LN dissection may increase the complete resection rate, but whether this approach improves the overall survival rate remains uncertain. A randomized study of two-field *vs* three-field LN dissection reported a significantly higher complication rate in three-field LN dissection, with no significant differences in recurrence or survival[10]. On the other hand, some non-randomized trials have reported a survival advantage associated with three-field LN dissection[11].

One limitation of surgery is that, at the time of diagnosis, two-thirds of patients with ESCC present with advanced, inoperable tumor stages and severe comorbidities. Another limitation is that resection margins are clearly defined in (at most) one-third of patients[12]. According to the Comprehensive Registry of Esophageal Cancer in Japan, 2006, the 5-year survival rate post-esophagectomy was 52% for patients with no residual tumor, but decreased to only 14% if residual tumors were present[9]. In addition, even if tumors were completely resected, the prognosis was poorer in patients with LN metastasis than in patients without LN metastasis; the 1-year, 3-year, and 5-year survival rates of patients with LN metastasis were 77%, 45%, and 35%, respectively[9]. These unsatisfactory outcomes have prompted investigation into multidisciplinary management involving CT, RT, and CRT, especially in the neoadjuvant setting.

STRENGTHS AND LIMITATIONS OF PREOPERATIVE THERAPY

Preoperative therapies can benefit ESCC patients in multiple ways. First, preoperative therapies can potentially downstage and degrade tumor size, and thus increase the possibility of complete resection. Second, they can eliminate possible hematogenous and/or lymphogenous micro-metastases from ESCC, and thereby limit postoperative disease recurrence. Third, undamaged blood and/or lymph vessels may permit more effective drug delivery to the tumor area.

One limitation of preoperative therapies is that surgical procedures are delayed in non-responders, exposing these patients to further metastatic spread. If this occurs, the effectiveness of preoperative therapy may be reduced, increasing postoperative morbidity and mortality. Currently, however, the relationship between preoperative therapy and postoperative morbidity and mortality remains controversial. Hirao *et al*[13] have reported that preoperative CT of JCOG9907 does not increase the risk of complications or hospital mortality after surgery for advanced thoracic ESCC. The meta-analysis conducted by Kranzfelder *et al*[14] revealed no evidence of increased mortality resulting from neoadjuvant CT and CRT. By contrast, randomized trials conducted by two independent groups did report increased postoperative mortality rates following neoadjuvant CRT[15,16].

NEOADJUVANT RT

The main purpose of preoperative neoadjuvant RT is to improve local control by down-sizing, if not eradicating, tumors in the involved LNs. Table 1 summarizes the

Table 1 Neoadjuvant radiotherapy treatment and outcomes for esophageal squamous cell carcinoma

Ref.	Year of publication	Histology	Treatment	*n*	Median survival (mo)	5-yr overall survival (%)	*P*
Launois *et al*[17]	1981	SCC	RT 40 Gy → Surgery	77	10	10	NS
			Surgery	57	12	12	
Gignoux *et al*[18]	1987	SCC	RT 33 Gy → Surgery	106	11	11	NS
			Surgery	102	11	10	
Arnott *et al*[19]	1992	AC/SCC (36%)	RT 20 Gy → Surgery	90	8	9	NS
			Surgery	86	8	17	
Nygaard *et al*[20]	1992	SCC	RT 35 Gy → Surgery	48		21[1]	0.080
			Surgery	41		9	
Wang *et al*[21]	1989	SCC	RT 40 Gy → Surgery	104		35	NS
			Surgery	102		30	
Cao *et al*[22]	2009	SCC	RT 40 Gy → Surgery	118		70[1]	0.005[1]
			Surgery	118		53	
Chu *et al*[23]	1994	SCC	RT 24-53 Gy → Surgery	40	11	10	NS
			Surgery → RT 45-53 Gy	42	11	10	

[1]3-yr overall survival. AC: Adenocarcinoma; NS: Not significant; RT: Radiation therapy; SCC: Squamous cell carcinoma.

results of six phase Ⅲ randomized trials, in which ESSC patients were treated by surgery supplemented with neoadjuvant RT or by surgery alone[17-22]. Two trials, conducted by Nygaard *et al*[20] and Cao *et al*[22], demonstrated a higher 3-year survival in the neoadjuvant RT + surgery group than in the group receiving surgery alone. The other four trials revealed no significant improvement of resectability or overall survival advantage. On the contrary, some of the studies reported a higher treatment-related mortality in the neoadjuvant RT + surgery group. One prospective randomized trial directly compared the therapeutic efficacy of preoperative *vs* postoperative RT in ESCC patients. This study found no difference in overall survival but reported a higher morbidity following preoperative RT[23]. A meta-analysis of 1147 cases, most of which were SCC, reported a slight trend in favor of neoadjuvant RT after a median follow-up period of 9 years, but the results were statistically insignificant (HR = 0.89, 95%CI: 0.78-1.01). In this study, the overall reduction in morbidity was 11% and the absolute survival benefit was 3% and 4% at 2 and 5 years, respectively[24]. In a SEER study of 1033 cases, 33% of whom presented with squamous cell carcinoma, demonstrated that the median overall survival and cause-specific survival were both significantly greater for patients who received neoadjuvant RT than for those receiving surgery alone (27 mo *vs* 18 mo and 35 mo *vs* 21 mo, respectively, $P < 0.0001$)[25]. However, since the SCC patients were not separately analyzed, the study presents no clear evidence that preoperative RT improves the survival of patients with potentially resectable ESCC. Thus, at present, preoperative neoadjuvant RT treatment is not recommended for ESCC patients.

NEOADJUVANT CT

In theory, preoperative CT is expected to down-stage the tumor prior to surgery, eradicate tumor micrometastases and reduce the risk of distant spread. In the 1990s, several randomized trials comparing neoadjuvant CT + surgery *vs* surgery alone were conducted on ESCC patients using CDDP, bleomycin, vindesin, 5-FU, and combinations of these drugs[20,26-29] (Table 2). However, none of these trials conclusively demonstrated the efficacy of neoadjuvant CT for patients with ESCC. Two large-scale randomized control studies have also been undertaken on this topic; the United Kingdom Medical Research Council esophageal cancer trial (OEO2) and Radiation Therapy Oncology Group (RTOG) 8911. OEO2 recruited 802 esophageal cancer patients to evaluate whether preoperative CT consisting of two cycles of CDDP and 5-FU followed by surgery improves survival compared with surgery alone[30]. The survival benefit was maintained with a HR of 0.84 (95%CI: 0.72-0.98; $P = 0.03$); the 5-year survival was 23% for the preoperative CT + surgery group, *vs* 17% for the surgery group. Although this study included both adenocarcinoma and squamous cell carcinoma, the treatment effect was independent of histological type[31]. However, the pattern of first disease progression was similar between the two treatment groups, in particular there was no clear trend toward fewer patients with distant metastases as first site of relapse in the preoperative CT + surgery group. The other large-scale study, RTOG8911, enrolled 443 patients with localized esophageal cancer, and compared the effect of CT plus surgery with that of surgery alone. This study showed no difference in overall survival between the two patient groups[32]. The reason for these disparate survival outcomes remains unclear, since both studies involved CDDP and 5-FU-based CT. However, a subgroup of the RTOG8911 study who responded objectively to neoadjuvant CT, when separately analyzed, showed significantly better survival outcomes than non-responding patients and all patients randomly assigned to surgery. Thus, effective CT will positively impact the survival of patients whose tumors respond to the administered chemotherapeutic agents. Importantly, an updated meta-analysis, which combined the data of OEO2 and RTOG8911, has proven that neoadjuvant CT confers a survival benefit over surgery alone in esophageal adenocarcinoma patients (HR = 0.83; 95%CI: 0.71-0.95; $P = 0.01$). However, CT supplements exerted no significant effect on the all-cause mortality of ESCC patients (HR =

Table 2 Neoadjuvant chemotherapy treatment and outcomes for esophageal squamous cell carcinoma

Ref.	Year of publication	Histology	Treatment	*n*	Median survival (mo)	5-yr overall survival (%)	*P*
Schlag[26]	1992	SCC	FU, CDDP → Surgery	22	10		NS
			Surgery	24	10		
Nygaard *et al*[20]	1992	SCC	CDDP, BL → Surgery	44	7	3[1]	
			Surgery	41	7	9	
Maipang *et al*[27]	1994	SCC	CDDP, BL, VI → Surgery	24	17	31[1]	NS
			Surgery	22	17	36	
Law *et al*[28]	1997	SCC	FU, CDDP → Surgery	66	17	40[1]	NS
			Surgery	69	13	13	
Ancona *et al*[29]	2001	SCC	FU, CDDP → Surgery	47	25	34	NS
			Surgery	47	24	22	
Kelsen *et al*[32]	2007	AC/SCC	FU, CDDP → Surgery	213	15	19	NS
(RTOG 8911)		(47%)	Surgery	227	16	20	
Allum *et al*[31]	2009	AC/SCC	FU, CDDP → Surgery	400	17	23	< 0.01
(OEO2)		(31%)	Surgery	402	13	17	
Ando *et al*[7]	2012	SCC	FU, CDDP → Surgery	164		55	0.01
(JCOG9907)			Surgery → FU, CDDP	166		43	

[1]3-yr overall survival. AC: Adenocarcinoma; BL: Bleomycin; CDDP: Cisplatin; FU: Fluorouracil; NS: Not significant; SCC: Squamous cell carcinoma; VI: Vinblastine; JCOG: Japan Clinical Oncology Group study.

0.92; 95%CI: 0.81-1.04, $P = 0.18$)[33].

Recently, the JCOG9907 study on resectable cStage Ⅱ/Ⅲ thoracic ESCC demonstrated that survival was significantly improved by preoperative CT with two courses of CDDP plus 5-FU followed by surgery, compared with postoperative CT. The 5-year overall survival was 43% and 55% in the postoperative and preoperative CT groups, respectively (HR = 0.73, 95%CI: 0.54-0.99, $P = 0.04$)[7]. The predecessor to this study, JCOG9204, had compared surgery + postoperative CT with surgery alone. These results indicate that additional postoperative CT treatment improved the disease-free survival of the entire cohort (from 45% to 55%, $P = 0.037$) and the 5-year overall survival in patients with LN metastases (52% *vs* 38% $P = 0.041$)[34]. Based on these data, preoperative CT followed by radical esophagectomy has become accepted in Japan as the standard therapeutic approach to resectable cStage Ⅱ/Ⅲ ESCC. However, we need to acknowledge that the trial design of JCOG9907 had some limitations[35]. In the postoperative treatment group, patients with LN metastasis negative cancer did not receive CT because JCOG9204 did not find a benefit for adjuvant CT in a subset analysis of LN metastasis-negative patients. Thus, this imbalance in treatment arms does not allow us to conclude that preoperative therapy is superior to postoperative therapy because not all patients in the postoperative CT arm received treatment. In addition, the primary end point of disease free survival was not met, yet overall survival was in favor of the preoperative group.

An optimal regimen of neoadjuvant CT against ESCC has yet to be established. The tumors of patients treated with neoadjuvant CT are potentially curable by surgery alone, and may progress to an inoperable stage while the patient is receiving preoperative CT. Thus, successful adjunct treatment requires a high response rate, or at least a high disease control rate. On the other hand, since esophagectomy is an invasive, surgically stressful procedure, preventing organ dysfunction and worsening of the patients' physical condition are also important. Especially, patients with ESCC frequently present with multiple organ disorders, because they are usually aged patients with a long-term history of smoking and alcohol use. In Japan, the JCOG9907 study has established a combination of CDDP and 5-FU as the standard regimen. However, the therapeutic efficacy of this regimen is by no means uniformly satisfactory; the response rate varies between 19% and 50%[7,12,26]. Thus, triplet CT, in which another drug is added to CDDP and 5-FP, has been intensively explored. A sole drug, docetaxel, has proven to positively supplement CDDP and 5-FP in randomized control trials. Docetaxel combined with CDDP and 5-FP (DCF therapy) is now regarded as a standard regimens for gastric or esophagogastric adenocarcinomas[36]. In addition, DCF is reportedly as effective as induction CT against head and neck squamous cell carcinoma, whose features are biologically similar to those of ESCC[37]. Regarding ESCC, exploratory trials of preoperative CT with DCF have demonstrated a high response rate (60%)[38,39]. Taken together, these results indicate DCF as a promising regimen of preoperative CT for ESCC.

NEOADJUVANT CRT

The role of neoadjuvant CRT has been debated for several decades. Various trials have compared the effects of neoadjuvant CRT in ESCC with those of surgery alone (Table 3). In most of these trials, CRT adjuvant treatment conferred no survival benefit; however, these trials can be criticized for inadequate trial design or small sample size. The Cancer and Leukemia Group B 9781 reported an overall survival enhancement in patients receiving neoadjuvant CRT; the 5-year overall survival was 39% in the neoadjuvant CRT + surgery group (95%CI: 21%-57%), *vs* 16% (95%CI: 5%-33%) in the surgery only group. Because this trial attracted few participants, it was closed,

Table 3 Neoadjuvant chemoradiotherapy treatment and outcomes for esophageal squamous cell carcinoma

Ref.	Year of publication	Histology	Treatment	*n*	Median survival (mo)	5-yr overall survival (%)	*P*
Nygaard *et al*[20]	1992	SCC	CDDP, BL + 35 Gy → Surgery	47	8	17[1]	
			Surgery alone	41	7	9	
Le Prise *et al*[47]	1994	SCC	CDDP, FU + 20 Gy → Surgery	41		47[2]	NS
			Surgery alone	45		47	
Apinop *et al*[48]	2004	SCC	CDDP, FU + 40 Gy → Surgery	35	10	24	NS
			Surgery alone	34	7	10	
Bosset *et al*[15]	1997	SCC	CDDP + 37 Gy → Surgery	143	19	7	
			Surgery	139	19	9	
Urba *et al*[49]	2001	AC/SCC	CDDP, FU, VI + 45 Gy → Surgery	50	17	16	NS
		(25%)	Surgery alone	50	18	30	
Heise *et al*[50]	2001	SCC	FU, LV, ET, CDDP + RT→ Surgery	33	20	26	
			Surgery alone	170	14	17	
Lee *et al*[51]	2004	SCC	CDDP, FU + 45 Gy → Surgery	51	28	55[3]	
			Surgery alone	50	27	57	
Burmeister *et al*[52]	2005	AC/SCC	CDDP, FU + 35 Gy → Surgery	128	22	17	NS
		(35%)	Surgery alone	128	19	13	
Natsugoe *et al*[53]	2006	SCC	CDDP, FU + 40 Gy → Surgery	22		57	NS
			Surgery alone	23		41	
Tepper *et al*[54]	2008	AC/SCC	CDDP, FU + 50 Gy → Surgery	30	54	39	0.002
(CALGB9781)		(75%)	Surgery alone	26	23	16	
Cao *et al*[22]	2009	SCC	CDDP, FU, MMC + 40 Gy → Surgery	118		73[1]	< 0.01
			Surgery alone	118		53	
Lv *et al*[55]	2010	SCC	CDDP, PTX + 40 Gy → Surgery	80	53	44	0.040
			Surgery alone	80	36	34	
Van Hagen *et al*[56]	2012	AC/SCC	CA, PTX + 41 Gy → Surgery	178	49	47	0.003
(CROSS)		(23%)	Surgery	188	24	34	

[1]3-yr overall survival; [2]1-yr overall survival; [3]2-yr overall survival. AC: Adenocarcinoma; BL: Bleomycin; CA: Carboplatin; CDDP: Cisplatin; DO: Doxorubicin; FU: Fluorouracil; ET: Etoposide; NS: Not significant; PTX: Paclitaxel; SCC: Squamous cell carcinoma; VI: Vinblastine; CALGB: Cancer and Leukemia Group B.

and hence is limited by small sample size (56 patients). Recently, a large-scale randomized trial (CROSS study) from the Netherlands has shown that preoperative CRT (carboplatin, paclitaxel, and RT 41.4 Gy) improves survival among patients with potentially curable esophageal or esophagogastric-junction cancer; the median overall survival was 49 mo in the CRT + surgery group, *vs* 24 mo in the surgery only group. Overall survival was also significantly better in the CRT + surgery group (HR = 0.66; 95%CI: 0.50-0.87; P = 0.003). Importantly, the benefit of neoadjuvant CRT was confirmed in an SCC subgroup (HR = 0.45; 95%CI: 0.24-0.84; P = 0.007). In addition, two meta-analyses have demonstrated that neoadjuvant CRT can improve the pathological response rate, local and regional control and the 3-year overall survival, compared with surgery alone[40,41]. In a recent meta-analysis of 9 randomized trials[14], neoadjuvant CRT delivered a clearly significant survival benefit to ESCC patients; the estimates of effect significantly favored neoadjuvant CRT (HR = 0.81, 95%CI: 0.70-0.95; P = 0.008). Moreover, neoadjuvant CRT did not alter the post-surgical morbidity and mortality rates.

FUTURES DIRECTIONS

In Japan, preoperative CT (FU + CDDP) followed by radical esophagectomy is the standard therapeutic approach to operable ESCC. However, systemic and regional recurrences are relatively common among patients treated by this approach. To overcome this problem, Japanese health authorities are currently reviewing their therapeutic strategies. In contrast to Japan, Western countries have adopted CRT as the standard therapeutic strategy. Whether preoperative CRT with radical surgery is effective for Japanese ESCC patients has yet to be established. Another promising regimen is preoperative triple-drug CT (involving docetaxel, CDDP and 5-FU). This background has initiated the JCOG1109 (NExT study) trial, a three-arm phase Ⅲ trial started in November of 2012. The aim of this study is to confirm whether docetaxel, CDDP + 5-FU is superior to CDDP + 5-FU, and whether CDDP + 5-FU is superior to CRT over CDDP + 5-FU, as preoperative therapies for ESCC[42]. Depending on the outcome of the JCOG1109 trial, the current ESCC therapeutic strategy might become altered in Japan. Importantly, the phase 2 study for neoadjuvant CRT (docetaxel, CDDP, 5-FU and concurrent RT) showed promising results; pathological complete remission (pCR) was found in 47%, and the 3- and 5-year survival rates were, respectively, 83% and 77% for pCR cases[43].

The limited improvements in treatment outcomes provided by conventional therapies have prompted us to seek innovative strategies for ESCC treatment; in particular, molecularly-targeted treatments. However, no promising results have been reported to date. The addition of an angiogenesis inhibiting drug (bevacizumab) to neoadjuvant CT with CDDP and 5-FU conferred the same benefit to ESCC patients as CDDP and 5-FU alone, the

latter administered to a historical control group[44]. The addition of bevacizumab and erlotinib to neoadjuvant CRT (paclitaxel/carboplatin/5-FU/radiation) similarly delivered no extra survival benefit to esophageal cancer patients, nor improved the pathologic complete response rate over similar regimens[45]. A phase Ⅱ study with cetuximab and radiation therapy for patients with surgically resectable esophageal carcinomas (Hoosier Oncology Group G05-92) has shown that cetuximab and radiation therapy results in a pathologic complete response rate (67% for squamous cell carcinoma) that seems at least comparable with that of CT and radiation therapy[46]. Regarding locally advanced ESCC, some phase Ⅲ studies are now recruiting patients to investigate new CT combinations, especially molecular-targeting reagents such as panitumumab, gefitinib, and cetuximab.

CONCLUSION

Currently, no international consensus on therapeutic strategy has been established for resectable thoracic ESCC. Western countries are focusing on neoadjuvant CRT followed by surgery or definitive CRT, while neoadjuvant CT and subsequent esophagectomy have become the standard therapeutic strategy in Japan. Many phase Ⅲ trials, such as JCOG1109, are underway across the globe. Hopefully, the large datasets generated from these trials will assist our understanding of preoperative therapy, and guide the establishment of a universal standard strategy for resectable ESCC.

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REVIEW

Modulators affecting the immune dialogue between human immune and colon cancer cells

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Abstract

The link between chronic inflammation and colorectal cancer has been well established. The events proceeding along tumorigenesis are complicated and involve cells activated at the cancer microenvironment, tumor infiltrating polymorphonuclears, immune cells including lymphocyte subtypes and peripheral blood mononuclear cells (PBMC), as well as tumor-associated macrophages. The immune cells generate inflammatory cytokines, several of them playing a crucial role in tumorigenesis. Additional factors, such as gene expression regulated by cytokines, assembling of tumor growth- and transforming factors, accelerated angiogenesis, delayed apoptosis, contribute all to initiation, development and migration of tumor cells. Oxygen radical species originating from the inflammatory area promote cell mutation and cancer proliferation. Tumor cells may over-express pro-inflammatory mediators that in turn activate immune cells for inflammatory cytokines production. Consequently, an immune dialogue emerges between immune and cancer cells orchestrated through a number of activated molecular pathways. Cytokines, encompassing migration inhibitory factor, transforming growth factor beta 1, tumor necrosis factor-α, Interleukin (IL)-6, IL-10, IL-12, IL-17, IL-23 have been reported to be involved in human cancer development. Some cytokines, namely IL-5, IL-6, IL-10, IL-22 and growth factors promote tumor development and metastasis, and inhibit apoptosis *via* activation of signal transducer activator transcription-3 transcription factor. Colon cancer environment comprises mesenchymal, endothelial and immune cells. Assessment of the interaction between components in the tumor environment and malignant cells requires a reconsideration of a few topics elucidating the role of chronic inflammation in carcinogenesis, the function of the immune cells expressed by inflammatory cytokine production, the immunomodulation of cancer cells and the existence of a cross-talk between immune and malignant cells leading to a balance in cytokine production. It is conceivable that the prevalence of anti-inflammatory cytokine production by PBMC in the affected colonic mucosa will contribute to the delay, or even to halt down malignant expansion. Targeting the interplay between immune and cancer cells by mediators capable to alter cytokine secretion toward increased anti-inflammatory cytokine release by PBMC and tumor associated macrophages, may serve as an additional strategy for treatment of malignant diseases. This review will focus on the inflammatory events preceding tumorigenesis in general, and on a number of modulators capable to affect colon cancer cell-induced production of inflammatory cytokines by PBMC through alteration of the immune cross-talk between PBMC and cancer cells.

Key words: Cytokines; Immune dialogue; Colon cancer; Peripheral blood mononuclear cells; Cross-talk

Core tip: The substantial number of studies that soundly demonstrated the close relationship between chronic inflammation and colon carcinogenesis has encouraged researchers to investigate the pathways interrelated with this process. The results point-out to various fac-

tors, molecules and genes that may jointly enhance or inhibit tumor development. The close linkage between immune and colon cancer cells resulting in a cross-talk between them with a consequent equilibrium in inflammatory cytokines release opens a new window for understanding the complicated stages of cancer initiation and progression. Moreover, the capability of emerging modulators to target the dialogue between immune and cancer cells indicates that immunomodulation may serve as a promising addition to the drug armamentarium for colorectal cancer.

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INFLAMMATION AND CANCER

Colon cancer is one of the common malignancies observed in clinical practice and it is one of the frequent causes of human death. No wonder therefore, that extensive efforts have been made, and are still carried on to enlighten the grounds providing suitable conditions for initiation, development, proliferation and spreading of this malignant process. Therefore, to mention even a part of the studies on the subject is beyond the scope of the present review. However, it seems reasonable to focus on a topic that has gained a wide interest, *i.e.*, the relation between chronic inflammation and cancer in general and colorectal malignant tumors in particular. Clinical observations based on the increased rate of colorectal cancer in patients with chronic colitis and Crohn's disease support this concept[1,2]. According to Rogler[3], thirty five percent of the patients suffering from ulcerative colitis for more than 35 years are at an increased risk for development of colorectal cancer, although population based studies point toward a lower risk. Factors, such as enhanced activation of the inflammatory cells by malignant cells and by the tumor microenvironment may initiate, and further promote cancer development[1,4]. Nguyen *et al*[5] have reported data suggesting that incitement of epithelial signal transducer activator transcription-3 (STAT3) in the inflamed colon plays a significant role in tumor progression by increasing mobilization and infiltration with $CD8^+$ lymphocyte population in the large intestine. Furthermore, according to the authors, activated STAT3 restrains the recruitment of regulatory Treg lymphocytes that possess the ability to suppress host immune responses with a subsequent enhancement of tumor progression. To make the issue more complicated, there are suggestions supporting the possibility of cancer-related inflammation, *i.e.,* the prospect that alterations inflicted by the tumor itself are those to provoke the inflammatory process[6]. Shigdar *et al*[7] emphasize the role of tumor associated immune cells that secrete cytokines capable to promote development of tumor stem cells. The activated stem cells from their part produce factors building a microenvironment ready to facilitate tumor growth and spreading. The connection between the immune activity of mononuclear cells and inflammation has been demonstrated by Leung *et al*[8] who have examined interleukin (IL)-12A and IL-22 cytokine production and the number of T helper cells from colon of patients with ulcerative colitis and Crohn's disease. The results showed an increased release of $IL\text{-}17^+$ and an elevated number of $CD4^+$ cells in patients with Crohn's disease compared to healthy individuals and patients with ulcerative colitis. On the other hand, patients with ulcerative colitis had decreased number of $IL\text{-}22^+$ cells. The role of bacteria in colon cancer etiology has been considered. Based on the fact that colonic mucosal cells from colorectal carcinoma are colonized by intracellular *Escherichia coli* (*E. coli*) and that the DNA repair gene *MUTYH* being blamed for cancer development is a homologue of *E. coli* gene mutY, Khan *et al*[9] have suggested that *mutY* gene and *E. coli* themselves might be involved in colorectal carcinoma development. Aggarwal *et al*[10] have reviewed in detail the links that build the chain of events leading to cancer and consist of pro-inflammatory substances that suppress apoptosis, enhance neovascularization and promote an increased activity of the immune system with a subsequent generation of pro-inflammatory cytokines. As for the relationship between chronic inflammation and carcinogenesis Basnet *et al*[11] divide the pro-inflammatory factors in two groups *i.e.*, external, that include pollutants, viruses, bacteria and even foods, as well as internal, comprising free radicals and the cytokines IL-1β, tumor necrosis factor (TNF)-α, NF-κB and NSAID-activated gene-1. It should be emphasized that cancer-associated inflammation plays not only an etiological role, but has also a therapeutic and even a prognostic potential. Thus, Laird *et al*[12] have shown that an inflammation score based on C-reactive protein level and albumin concentration on one hand and patient's performance status on the other hand, predicts fairly well the survival of patients with advanced stage cancer. A review on the relationship between innate immunity, inflammation and cancer, detailing the function of inflammatory cytokines as mediators of inflammation-related carcinogenesis has been reported by Lin *et al*[13].

PERIPHERAL BLOOD MONONUCLEAR CELLS AND CYTOKINE PRODUCTION

In regard to the link between chronic inflammation and carcinogenesis, the mononuclear cells appear to be persuasive warriors expressing a vivid phagocytic capacity[14], to encompass Toll-like receptors able to recognize pathogen molecules and to be capable to modulate both innate and adoptive immune responses[15-17]. Studies have shown that the number of Th1 and Th17 cells is increased in patients with inflammatory bowel disease and correlates well with its severity[18], whereas the number of $CD14^+$ and $CCL11^+$ mononuclear cells has been found to be

increased in colonic biopsies from patients with ulcerative colitis[19]. According to van Dooren *et al*[20] there is a difference between cytokine production by whole blood with CCL11, IL-23 and IL-12p40 solely presented, and lipopolysaccharide (LPS) stimulated peripheral blood mononuclear cells (PBMC) producing IL-20, vascular endothelial growth factor (VEGF) and GM-colony stimulating factor (CSF). Endogenous type interferon 1 (IFN-1) released by colon mononuclear cells in mice with T-cell colitis has been demonstrated to be essential for stimulated production of the anti-inflammatory cytokines IL-10, IL-1ra and IL-27[21]. Inflammasomes are closely related to chronic inflammation since they act as activators of IL-1β and IL-18 release by PBMC[22,23]. Moreover, activation of inflammasomes may stimulate cancer development[24]. The ability of PBMC to produce inflammatory cytokines plays a major role in the modulation of chronic inflammatory responses associated with carcinogenesis. This particular function is promoted by various factors, such as amorphous silica particles capable to increase IL-1β and IL-8 production[25], and cortisol that have been shown to suppress secretion of IL-1β, IL-6, IL-17 and G-CSF[26]. Recently, Veinalde *et al*[27] have reported that double stranded RNA has the capacity to induce PBMC to produce a considerable number of both pro- and anti-inflammatory cytokines. Metal particles, such as titanium and stainless steel originating in patients with implanted medical devices have been shown to enhance increased production of IL-6 and IL-1 by PBMC, but to lower the level of TNF-α[28]. Even mental conditions, such as post-traumatic stress disorders, may affect the immune activity of PBMC causing an increased spontaneous release of the pro-inflammatory cytokines IL-1β and TNF-α[29]. Finally, it should be noted that there is no obligatory correlation between the level and type of cytokines produced by PBMC and those in the whole blood[20].

COLON CANCER CELLS AND CYTOKINE PRODUCTION

Concerning the role of chronic inflammatory process as a basis for carcinogenesis, the question if colon cancer cells possess the capacity for cytokine production comes up. In that sense the reports in the literature are rather scarce. It is our experience that HT-29 and RKO cells from human colon cancer lines do not produce cytokines unless they are exposed to various stimuli. Yoshimoto *et al*[30] have shown that HT-29 carcinoma cells stimulated with LPS, IFNγ and epithelial growth factor (EGF) expressed a modulation of the Hedgehog (Hh) pathway signaling. Hh agonists exerted a decrease of IL-8 and monocytic chemotactic protein-1, compared to its antagonists such as dopamine, LPS, IFNγ and EGF. The connection between chronic inflammation, cytokine release and colon cancer has been reviewed by Klampfer *et al*[4] and by Lin *et al*[13]. According to the authors, the inflammatory process is initiated and further driven to carcinogenesis by soluble factors and cytokines released by both cancer-and recruited immune cells, including those at the tumor environment. TNF-α, IL-6, IL-12 and IL-23 from the group of pro-inflammatory cytokines are of particular importance, since they are involved not only in maintaining the inflammatory process, but they promote tumor cell survival by exerting an anti-apoptotic activity, induce angiogenesis and are crucial for further tumor development and tumor cell migration.

IMMUNE CROSS-TALK BETWEEN PBMC AND CANCER CELLS

Accepting the presumption that carcinogenesis is closely linked to chronic inflammation, it is conceivable that immune, stromal and mast cells mobilized to an affected area will interfere with tumor cells and will establish an immune dialogue resulting in a prompt release of a number of inflammatory cytokines, as it is graphically shown in Figure 1. It has been reported that unstimulated PBMC release a small amount of inflammatory cytokines[31]. On the other hand, PBMC exposed to HT-29 or RKO cells from human colon cancer lines or their supernatants were able to release both pro-and anti-inflammatory cytokines, in some cases in a dose-dependent matter. However, the release of the pro-inflammatory cytokines TNF-α, IL-1β and IFNγ was more pronounced[31]. It is notable that direct exposure of PBMC to cancer cells resulted in higher cytokine secretion compared to cytokine levels released by PBMC incubated with cancer cells' supernatants, a finding similar to that observed by Ma *et al*[32] in co-cultures of PBMC with gastric cancer cells. In their hands the release of transforming growth factor (TGF)-β by PBMC was markedly increased when they were co-cultured with gastric carcinoma cells. Cytokines secreted during immune-cancer cells communication may affect the process of carcinogenesis. Combination of the pro-inflammatory cytokines IL-32 and TNF-α restrained the growth of HCT116 and SW620 human colon cancer cells by inhibition of TNF-α dependent DNA synthesis[33]. PBMC co-cultured with cells from an AGS human gastric epithelial line expressed a decreased TGF-β1 and an increased TGF-β2 cytokine secretion. On the other hand, incubation of PBMC with cells from a gastric cancer line (MKN45) caused enhancement in TGF-β1 production, a cytokine known to move forward cancer development. It is of interest that cancer cells incubated with PBMC showed an increase in TGF-β1 mRNA level up to 3-fold higher than cancer cells cultured alone[32]. The way PBMC and cancer cells create an immune dialogue is intriguing. Studies have shown that intercellular communication between tumor-associated leukocytes and malignant cells proceeds through exosomes released from tumor cells and results in enhanced production of pro-inflammatory cytokines and metalloproteinases[34]. Redzic *et al*[35] have underlined the capacity of extracellular vesicles purified from various cancer cell lines to stimulate PBMC to release a number of tumor promoting factors including IL-6. The role of autophagy in inflection of both innate

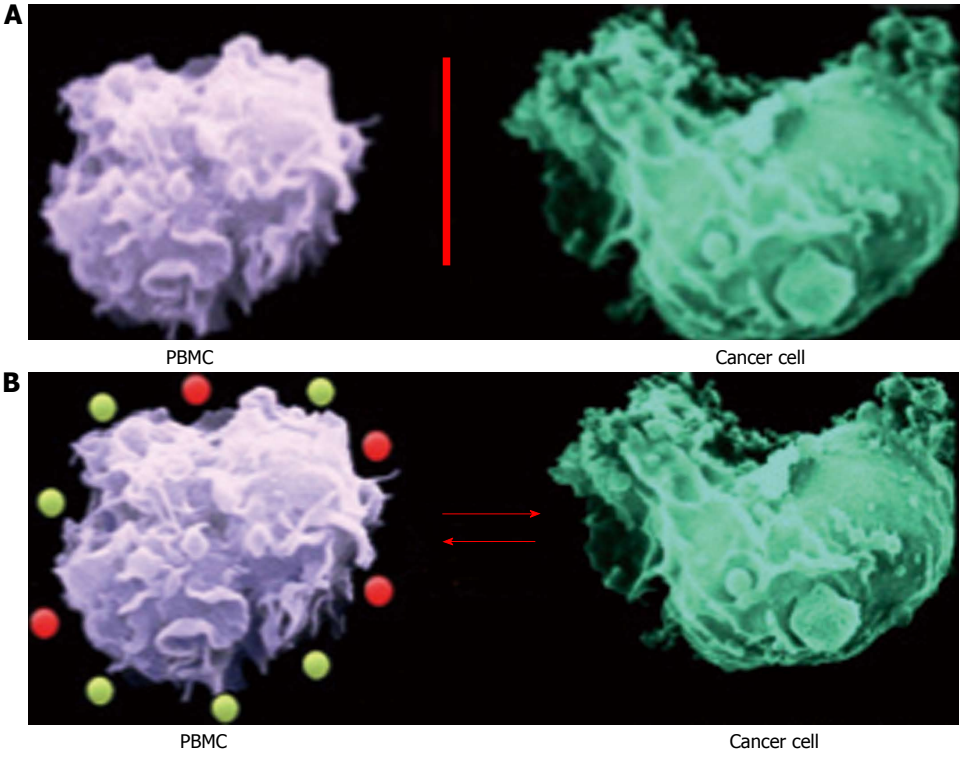

Figure 1 Interrelationship between peripheral blood mononuclear cells and human colon cancer cells. A: Unstimulated peripheral blood mononuclear cells (PBMC) do not release significant amount of inflammatory cytokines. B: Following direct contact with cancer cells PBMC are stimulated for pro-inflammatory (red dots) and anti-inflammatory (yellow dots) cytokine production.

and adaptive immunity is gaining importance. Autophagy modulates production of the pro-inflammatory cytokines IL-1β and IL-18 that consecutively enhance the functional expression of B and T lymphocytes, as well as that of the IL-2 receptor[36]. Reduced expression of IL-1β, IL-18 and IL-21 in mice with colitis-associated cancer resulted in achievement of a higher clinical score[37,38]. Conversely, cytokines released by immune cells participated in regulation of autophagy. Thus, IFNγ being a Th1 helper cytokine induced enhanced autophagy in macrophages, whereas IL-4 and IL-13, which are Th2 cytokines, exerted an inhibitory effect[39]. Similar findings have been reported by Schmeisser *et al*[40] who have shown that IFNγ and TGF-β enhance autophagy in human cancer lines derived from uterus cervix, breast, glioblastoma and alveolar carcinoma. The role of autophagy in regulation of immune responses has been described in details by Valdor *et al*[39].

MODULATORS OF THE CROSS-TALK BETWEEN IMMUNE AND COLON CANCER CELLS

It has been shown that during the inflammatory process, the vicinity of the tumor comprises tumor-associated macrophages and a significant number of white blood cells producing various cytotoxic mediators, enzymes linked with tumor development and cytokines, such as IL-1, IL-6, IL-8 and IFNs[41]. A considerable number of drugs, vitamins, nutrients and spices have been shown to exert anti-cancerous effect *via* various pathways. Relying on observations that immune cells maintain chronic inflammatory processes by cytokine release, and the ability of cancer cells to alter the type and level of cytokine production following direct cell contact, the question arises if intercellular communication may be directed by immune modulators in a way capable to increase production of anti-inflammatory cytokines, as schematically presented in Figure 2. Table 1 summarizes our experience and findings with modulators that target the communication between immune and cancer cells.

Aspirin

It is conceivable that anti-inflammatory drugs may play a major role in both abolishing inflammation and restraining cancer development. Indeed, a substantial number of experimental data indicates that non-steroidal anti-inflammatory drugs (NSAID) may inhibit colon cancer development. The anticancer activity of most of them is based on their selective inhibitory effect on cyclooxygenase-2 (COX-2) activities, that play a crucial role in colon cancer development and progress[42]. In that sense, aspirin has drawn particular attention since it expresses an inhibitory activity on both COX-1 and COX-2 enzymes. However, studies have showed that NSAID anti-cancer properties may proceed also through COX-independent pathways, such as inappropriately Delta1/Notch1 signal transduction pathway, and upregulation of *NAG-1* gene that is a member of the TGF-β superfamily[42-44]. Bergman *et al*[45] have found that addition of aspirin to PBMC

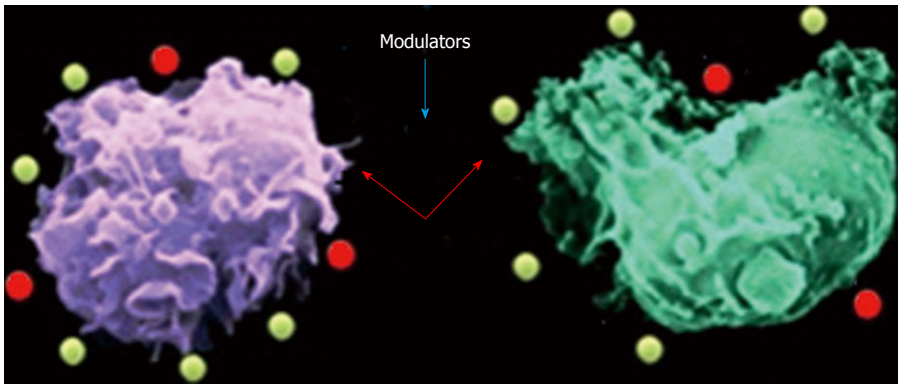

Figure 2 Schematic presentation of the way immune modulators modify the cross-talk between peripheral blood mononuclear cells and cancer cells. Following alteration of the immune dialogue between these two cell types, the modulators inhibit cancer cell-stimulated peripheral blood mononuclear cells (PBMC) to generate pro-inflammatory cytokine (red dots), acting as cancer promoters.

Table 1 Modulators acting on cross talk-induced cytokine secretion

	Ref.	Pro-inflammatory cytokines				Anti-inflammatory cytokines	
		IL-1β	IL-6	TNFα	IFNγ	IL-1ra	IL-10
PBMC + colon cancer cells	Bessler *et al*[31] (2010)	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑
Modulator							
Aspirin	Bergman *et al*[45] (2011)	↑ slightly	↓	ND	↓/↑[1]	0/↓[1]	↓
Colchicine	(Submitted for publication)	↑	0/↑[1]	↓	ND	0	↓/↑[1]
Statins	Bergman *et al*[66] (2011)	↓	0	ND	↓	↓	↓
Caffeine	Bessler *et al*[75] (2012)	0	0	↓	↓	↓	↓
Resveratrol	Bergman *et al*[82] (2013)	↓	↑/0[1]	↓	↓	↓	↓
Curcumin	Bessler *et al*[92] (2012)	↓	↓	↓	ND	↓	↓
Vit. D3	Bessler *et al*[99] (2012)	↓	0	↓	ND	0	↓

[1]Respresents results for HT-29-induced/RKO-induced cytokine secretion by PBMC. ND: Not determined; 0: No change in cytokine secretion; PBMC: Peripheral blood mononuclear cells; IFN: Interferon; TNFα: Tumor necrosis factor-α; IL-6: Interleukin-6; Vit. D3: Vitamin D3.

co-cultured with HT-29 or RKO cells from human colon cancer lines, affected the immune equilibrium between immune and cancer cells by inducing inhibited production of the pro-inflammatory cytokines IFNγ and IL-6. Notably, the secretion of anti-inflammatory cytokines was somewhat depressed. Skeen *et al*[46] have stressed the role of the cross-talk between the anti-inflammatory cytokine TGF-β1 and factors critical for colorectal tumorigenesis. A decreased TGF-β1 expression is linked to both increased inflammation and colorectal cancer evolution. The expansion of colon-cancer is closely related to the pro-inflammatory cytokines IL-6, IL-8, TNF-α, and VEGF. Aspirin has been shown to reduce the pro-inflammatory IL-6 expression with a subsequent reduction of CRP, an important protein for the maintenance of chronic inflammation[47]. Since cancer cells' apoptosis and death are mediated through TNF-α and IL-1-induced transcription factor NF-κB activation, aspirin may increase the apoptotic rate of the malignant cells by inhibited expression of these cytokines[48]. On the other hand, NF-κB activation can promote production of pro-inflammatory cytokines in colon cancer cells and particularly IL-8 with a further enhancement of the gastrointestinal inflammation that may explain the undesirable effect of aspirin on the alimentary system[49]. Lang *et al*[50] have found that monocytes incubated with supernatants from human carcinoma cell lines showed a down-regulation of the chemokine receptor CCR5, and beta 2-integrin Mac-1, resulting in impaired monocyte migration and adhesion, functions important for exertion of their anti-cancer activity. The authors have observed that administration of a selective COX-2 inhibitor, rofecoxib, to cancer patients improved markedly their reduced monocyte CCR5 levels and migration capacities. Using SW480 colorectal cells, Lai *et al*[51] have reported that aspirin, in a concentration-dependent manner, was able to inhibit their proliferation and promote cancer cell apoptosis and necrosis by cell arrest at the G_0/G_1 phase. The proportions of cells at the S- and G_2/M phases was decreased.

Colchicine

Colchicine is an anti-inflammatory agent, known since long as the drug of choice for management of gout. It is gaining increased interest for treatment of other conditions, such as familial Mediterranean fever and Behçet disease. It has been shown that its anti-inflammatory effect proceeds *via* inhibition of the pro-inflammatory cytokines IL-1 and IL-1β release[52]. Moreover, by restrain of the NF-κB pathway and blocking cell mitosis, colchicine may exert an inhibitory effect on tumorigenesis. Addition of colchicine to human LPS stimulated PBMC exerted a disruptive effect on cellular microtubules with a consequent increased IL-1β and a decreased TNF-α release[53]. In a recent study it has been shown that colchicine added to co-cultures of PBMC with either HT-29 or RKO human colon cancer cells promoted cancer cells-stimulated PBMC to produce IL-1β and to inhibit the release of TNF-α and IL-10[54]. It is conceivable that colchicine may

act on tumor development by modulation of the immune balance between immune and cancer cells with a subsequent interference in production of inflammatory cytokines by cancer cells-activated PBMC. A similar tumor cell-macrophage cross-talk has been observed in other studies. TNF-α released from colon cancer cells stimulated TNF-α and CSF-1 production by mouse macrophages indicating the existence of an immune intercellular communication between these cell types[55]. Furthermore, it has been shown that monocytes interacting with cancer cells differentiate into tumor-associated macrophages that may be associated with angiogenesis and metastasis[56].

Statins

Although statins, branded as 3-hydroxy-methylglutaryl coenzyme (HMG-CoA) reductase inhibitors, have been introduced as potent anti-cholesterol agents in cardiovascular diseases, a substantial number of studies indicate that these drugs possess additional attributes including anti-inflammatory[57,58] and anti-cancer activities[59,60]. It is notable that statins enhance cytokine production by acting directly on PBMC. The hydrophobic statins lovastatin and simvastatin increased the production of IL-1β and decreased IL-2 and IFNγ secretion[61]. Simvastatin, atorvastatin, fluvastatin and pravastatin reduced IL-6 production by human PBMC co-cultured with human vascular smooth muscle cells by 53%, 50%, 64% and 60% respectively[62]. Simvastatin induced increased production of IL-18, TNF-α and IFNγ by human PBMC[63]. Bessler *et al*[64] have shown that the effect of statins on malignant cell proliferation depends on their dosage, physiochemical properties and the type of cancer cells. Both hydrophilic and hydrophobic statins inhibited proliferation, but not apoptosis in cells from HuCC human colon carcinoma line. However, this effect differed when statins were incubated with EHEB, K562 and Raji-cells (B chronic lymphocytic leukemia, human erythroleukemia, and Burkitt lymphoma cell lines, respectively). EHEB and K562 cell proliferation was inhibited by hydrophobic but not by hydrophilic statins. The hydrophobic statins enhanced cell apoptosis in the hematological lines. Similar observations have been reported by Kato *et al*[60] who have found that hydrophobic, but not hydrophilic statins, induced apoptosis in cells from gynecological cancers expressing high levels of HMG-CoA reductase. Simvastatin caused a decreased release of IL-6 and IL-8 from HT-29 and Caco-2 cells derived from colorectal cancer lines[65]. The capacity of statins to modulate inflammation-induced colon cancer proliferation by alteration of the equilibrium between pro- and anti-inflammatory cytokines secreted by tumor cells-stimulated PBMC is of particular interest. Statins added to PBMC stimulated for cytokine secretion by direct contact with HT-29 or RKO cells from human colon carcinoma lines induced a decreased expression of the anti-inflammatory cytokines IL-1ra and IL-10[64,66].

Nutrients

Nutrients and naturally-occurring phytochemicals are gaining positive reputation based on their beneficial properties on health and their carcino-preventive capacities[67]. The number of studies on the potential inhibitory activity of these substances on colorectal cancer development is rapidly mounting; hence to encompass the mechanisms by which all of them influence tumorigenesis is beyond the scope of the present review. Having a personal experience with a few of them, we therefore will concentrate on phytochemicals and nutrients with substantiated anti-inflammatory and anti-cancer activities.

Caffeine

The xanthine alkaloid caffeine is the main compound of coffee, one of the most popular beverages renowned for its long history. Apart from its beneficial effect on a lengthy list of diseases[68], studies demonstrate that coffee possesses anti-cancer properties. Clinical studies indicate that prolonged coffee consumption abolishes the risk for colorectal cancer[69,70]. The ways caffeine exerts its carcino-preventive effect have been reviewed in details[71,72]. It is noteworthy that caffeine is involved in modulation of the immune system. It has been reported that addition of caffeine to concanavalin A-stimulated mouse lymphocytes inhibited the generation of TNF-α, IFNγ and IL-2[73]. Caffeine, being a potent adenosine receptor antagonist, has been found to be able to decrease TNF-α release from LPS stimulated cord blood monocytes[74]. Due to its inhibitory activity on adenosine receptors caffeine has been capable to alter the stability of hypoxia-inducible factor 1-alpha, VEGF and IL-8 expression in tumor cells[72]. Bessler *et al*[75] have suggested an additional mechanism based on the capacity of caffeine to alter the immune balance between PBMC and HT-29 or RKO cells derived from human colon cancer lines. The authors have observed that caffeine inhibited secretion of the pro-inflammatory cytokines TNF-α and IFNγ by PBMC stimulated for cytokine production through a direct contact with cancer cells. The fact that caffeine did not enhance cytokine production by PBMC co-cultured with both types of cancer cells and that supernatants derived from cancer cells incubated with caffeine did not affect this activity, brought the authors to the conclusion that caffeine, at least *in vitro*, exerts its anti-inflammatory and anti-cancer effects *via* increasing anti-inflammatory cytokine production by PBMC, but only subsequent to a direct contact between immune and cancer cells.

Resveratrol

Resveratrol, a phenolic compound present in grapes and their seeds has been shown to exert a favorable effect on a number of diseases[76]. Moreover, studies have shown that it may act as an inhibitor of cancer development[77-79]. The mechanisms explaining resveratrol's anti-inflammatory properties have been reviewed by de la Lastra *et al*[80]. According to Richard *et al*[81] resveratrol added to stimulated PBMC brought to an impaired early expression of IL-8 and TNF-α. Bergman *et al*[82] have reported that resveratrol, while added to non-stimulated PBMC expressed a minimal activity for cytokine production, it did not enhance cytokine release by HT-29 and RKO human

colorectal cells. However, resveratrol added to PBMC co-cultured with cancer cells inhibited the release of IL-1β, IL-6, TNF-α, IFNγ, IL-1ra and IL-10. Here again, this is an example of the way a modulator targets the immune dialogue between immune and cancer cells by affecting inflammatory cytokine production with a consequent impact on tumorigenesis. It should be stressed that a substantial number of dietary polyphenols have been found to express anti-inflammatory and anti-cancer properties. Thus, ellagic acid present in pomegranates was shown to reduce the expression of NF-κB, COX2, TNF-α and IL-6 and to exert both anti-inflammatory and carcinopreventive effects[83,84]. Polyphenol-rich apple diet administered to rats inhibited TNF-α, iNOS, IL-1β and IL-6 mRNA expression in the colon and diminished the number of CD68 cells[85]. Diet supplemented with cocoa polyphenols feed to rats with experimentally induced colon inflammation decreased the level of the nuclear NF-κB and of the pro-inflammatory enzyme COX-2[86]. The role of natural products and their phytochemicals in modulation of molecular pathways involved at various stages of tumorigenesis has been summarized by Rajamanickam *et al*[87].

Curcumin

The natural spice curcumin, extracted from the Curcuma longa plant has been recognized as an effective anti-inflammatory and anti-cancer agent[11,88]. As an inhibitor of tumorigenesis, curcumin acts as an anti-oxidant, apoptotic promoter and by expressing antifungal and immunomodulatory activities. As an immunomodulator curcumin is capable to modify T and B cell activities and to downregulate the release of the pro-inflammatory cytokines TNF, IL-1, IL-2, IL-6, IL-8, and IL-12 *via* NF-κB inactivation[89]. It has been reported that curcumin modulates DNA methylation in HCT116, HT-29 and RKO colorectal cancer lines[90]. Curcumin enhances the differentiation of myeloid-derived suppressor cells that are actively involved in tumor angiogenesis, tumor growth and metastasis[91]. Administration of curcumin to a mouse-colon cancer allograft model resulted in a decreased percentage of myeloid-derived suppressor cells in the peripheral blood and organs, reduced IL-6 levels and significantly inhibition of tumor growth. Similar findings were obtained when curcumin was added to myeloid-derived suppressor cells co-cultured with cancer cells. Bessler *et al*[92] have reported that curcumin caused a dose-dependent inhibition of the pro-inflammatory cytokines TNF-α, IL-1β and IL-6 produced by HT-29 or RKO stimulated human PBMC. The generation of IL-1ra, IL-10 and the proliferation of the cancer cells from both lines were also inhibited. These results were observed after co-culture of immune and cancer cells, indicating the likelihood of a direct interaction between immune and cancer cells, a plausible mechanism for explaining the inhibitory effect of curcumin on tumorigenesis. It is not wonder therefore, that efforts are ventured to synthesize compounds that are more efficient and less toxic than curcumin for augmentation the possibilities for colon cancer therapy[93].

1α, 25-Dihydroxyvitamin D3

Recently, a number of studies indicate that vitamin D (vit. D) exerts a protective action against colon cancer by several mechanisms, such as modifying cancer angiogenesis, cell differentiation, and proliferation, as well as apoptosis[94]. Moreover, due to presence of vit. D receptors in the PBMC the vitamin plays a significant role as an immune system regulator[95]. Mice lacking vit. D receptors developed inflammatory bowel disease and their $CD4^+$ cells produced more IFNγ and less IL-2, IL-4 and IL-5 compared to cells from wild type animals[96]. Mice that spontaneously developed symptoms of inflammatory bowel disease due to IL-2 deficiency showed reduced mortality after vit. D supplementation[97]. It should be stressed however, that IL-6 and TNF-α, two pro-inflammatory cytokines that are crucial for development of inflammatory bowel disease and colorectal cancer, may impair the anti-inflammatory activity of vit. D[98]. The prospect that vit. D may affect carcinogenesis by interfering with the immune relationship between immune and cancer cells has been examined by Bessler *et al*[99]. Incubation of PBMC with either HT-29 or RKO cells induced a marked enhancement of both pro- and anti-inflammatory cytokine production. Vit. D added to the incubation mixture containing PBMC and cells from both colon carcinoma lines caused a significant inhibition of TNF-α and IL-6 generation and to a lesser extent of the IL-10. In view of the key role of both TNF-α and IL-6 in maintaining chronic inflammation and promoting colorectal tumorigenesis, these findings suggest the existence of an additional mechanism for explaining the beneficial role of vit. D as an anti-cancer agent.

It should be underscored that the above-mentioned compounds illustrate a list of potential anti-cancer drugs, nutrients and spices that are gaining increased appreciation as modulators of the immune interaction between PBMC and cancer cells, and offer therefore an additional therapeutic opportunity for malignant diseases.

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RETROSPECTIVE STUDY

Prognostic value of baseline FDG uptake on PET-CT in esophageal carcinoma

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Abstract

AIM: To evaluate the influence of baseline maximum standardized uptake value (SUV_{max}) on survival in a cohort of patients, undergoing positron emission tomography-computed tomography (PET-CT) scan for esophageal carcinoma.

METHODS: The pre-treatment SUV_{max} numeric reading was determined in patients with confirmed esophageal or junctional cancer having PET-CT scan during the time period 1st January 2007 until 31st July 2012. A minimum follow up of 12 mo was required. Patients were subdivided into quartiles according to SUV_{max} value and the influence of SUV_{max} on survival was assessed using univariate and multivariate analysis. The following pre-treatment factors were investigated: patient characteristics, tumor characteristics and planned treatment.

RESULTS: The study population was 271 patients (191 male) with esophageal or junctional carcinoma. The median age was 65 years (range 40-85) and histologic subtype was adenocarcinoma in 197 patients and squamous carcinoma in 74 patients. The treatment intent was radical in 182 and palliative in 89 patients. SUV_{max} was linked to histologic subtype (P = 0.008), tumor site (P = 0.01) and Union for International Cancer Control (UICC) stage (P < 0.001). On univariate analysis, prognosis was significantly associated with SUV_{max} (P = 0.001), T-stage (P < 0.001) and UICC stage (P < 0.001). On multivariate analysis, only T-stage and UICC stage remained significant.

CONCLUSION: Pretreatment SUV_{max} was not a useful marker in isolation for determining prognosis of patients with esophageal carcinoma.

Key words: Esophageal neoplasms; Fluorodeoxyglucose F18; Positron emission tomography; Positron emission tomography-computed tomography; Prognosis

Core tip: Positron emission tomography-computed tomography (PET-CT) is integral to the staging of esophageal cancer. It is unclear whether the value of PET-CT extends beyond the identification of metastatic disease. The influence of PET-CT maximum standardized uptake value (SUV_{max}) on prognosis was determined for 271 patients. Although SUV_{max} was closely linked to disease stage, it did not exert an independent effect and was not a useful prognostic marker.

Al-Taan OS, Eltweri A, Sharpe D, Rodgers PM, Ubhi SS, Bowrey DJ. Prognostic value of baseline FDG uptake on PET-CT in esophageal carcinoma. *World J Gastrointest Oncol* 2014; 6(5): 139-144 Available from: URL: http://www.wjgnet.com/1948-5204/full/v6/i5/139.htm DOI: http://dx.doi.org/10.4251/wjgo.v6.i5.139

INTRODUCTION

Positron emission tomography (PET) is an important component in the staging algorithm for patients with cancers of the esophagus and gastroesophageal junction[1,2]. At some centers, it is employed early in the staging pathway with all patients being assessed by this modality. In other centers, it features later in the staging pathway, only being utilized if computed tomography (CT) and endoscopic ultrasound demonstrate potentially resectable tumor characteristics[1,2].

Its principal application is in the identification of occult metastatic disease, not identified on CT imaging, or in the confirmation of high fluorodeoxyglucose (FDG) uptake in suspicious areas on CT imaging[1].

We have previously shown that PET-CT influences the treatment decision overall for 10% of patients with esophageal cancer, and for 26% of patients free of definite metastatic disease after initial CT imaging[2].

The maximum standardized uptake value (SUV_{max}) is a measure of the relative metabolic activity of the cancer. A recent meta-analysis confirmed the close link between the SUV_{max} and both tumor stage and prognosis[3]. Whether the SUV_{max} exerted an independent effect, unrelated to known clinical prognostic markers was unclear.

The majority of studies have assessed selected patient groups, typically only those receiving one form of treatment such as chemoradiotherapy, palliative chemotherapy or surgery (with or without neoadjuvant chemotherapy)[4-24]. It is likely that this has resulted in clustering of SUV_{max} values. Only four smaller studies have assessed the influence of SUV_{max} in unselected patients undergoing PET-CT[8,14,21,24]. Those studies concluded that SUV_{max} was significantly associated with prognosis but that this was not independent of existing clinical markers such as Union for International Cancer Control (UICC) stage.

The aim of the current study was to assess whether the SUV_{max} provided additional prognostic information, over and above the UICC stage and known clinical prognostic markers in a large cohort of unselected patients.

MATERIALS AND METHODS

The use of anonymized patient information was approved by the Institutional Clinical Audit and Effectiveness Board. Individual patient consent was not required as no change in patient management was effected for the purposes of this audit.

The study was a retrospective review of all patients undergoing PET-CT during the time period 1st January 2007 to 31st July 2012. At our institution, PET-CT became incorporated into the staging algorithm of routine clinical practice in November 2006. Patients undergoing PET-CT after 31st July 2012 were not included, so that a minimum patient follow up time of 12 mo would be obtained.

All patients with a diagnosis of esophageal or gastric cancer are discussed at a weekly multi-disciplinary meeting and treatment intention and schedule determined. The staging algorithm has previously been published[2].

The 7th edition of the UICC stage was determined by consensus decision at the multi-disciplinary meeting based upon pre-treatment imaging.

PET-CT

During the years 2006-2008 coregistered PET-CT was performed using a General Electric Discovery ST PET-CT scanner with eight-slice CT scan, producing fused single image scans. Since 2008, imaging has been performed using a Siemens Biograph TruePoint PET-CT scanner. Half-body PET acquisition was obtained (from eyes to knees). Patients were fasted for 6 h prior to injection with 350-420 MBq of ^{18}F-FDG (4.5 MBq/kg) that was administered to patients lying supine in a quiet and warm environment. Whole-body two-dimensional image acquisition was obtained 60 min after injection of 18F-FDG using a 128 × 128 matrix. Fused PET-CT images were single reported with quality assurance validation of 10% of scans. The diagnostic CT and previous imaging was available at the time of reporting. The threshold for the diagnosis of metastatic disease on PET-CT was a standardized uptake value in excess of 2.5.

The influence of patient characteristics (age and sex), tumor characteristics (tumor location, histologic subtype, T stage, N stage and UICC stage), planned treatment strategy and baseline SUV_{max} on PET-CT were investigated using univariate analysis. Significant variables were then investigated using Cox regression analysis.

Parametric data were analyzed using the unpaired *t*-test and non-parametric data were analyzed using the Mann-Whitney and Kruskal-Wallis test. Statistical analysis was performed using SPSS software version 15 (SPSS, Chicago, IL, United States). Significance was assumed at the 5% level.

RESULTS

The study population comprised 271 patients (191 males) of median age 65 years (range 40-85). Primary tumor location was upper esophagus in 13 patients, middle esophagus in 50 patients, lower esophagus in 136 patients and gastroesophageal junction in 72 patients. Histologic subtype was adenocarcinoma in 197 patients and squamous cell carcinoma in 74 patients.

Distribution of UICC stage was as follows: Stage 0 or 1 (45 patients), Stage 2 (50 patients), Stage 3 (99 patients) and Stage 4 (77 patients). Stage 4 disease was defined on the basis of distant metastatic disease in 31 patients and on the basis of celiac axis lymphadenopathy in 46 patients. Lymphadenopathy anterior to the left gastric pedicle was defined as locoregional disease as this would be routinely within the field of surgical resection. Lymphadenopathy posterior to the left gastric pedicle was defined as celiac axis lymphadenopathy and would not be included in the field of surgical resection.

Of note, there was no significant difference in the SUV_{max} readings obtained during the two time periods,

Table 1 Influence of patient characteristics on maximum standardized uptake value and survival

Factor	Mean SUV_{max} (95%CI)	Median survival in days (95%CI)
Sex		
Male (n = 191)	11.4 (10.5, 12.3)	566 (491, 641)
Female (n = 80)	12.1 (10.2, 14.0)	884 (403, 1364)
	P = 0.950	P = 0.05
Age in years		
Age ⩽ 65 (n = 136)	11.5 (10.5, 12.5)	575 (456, 694)
Age > 65 (n = 135)	11.7 (10.4, 13.1)	586 (418, 754)
	P = 0.770	P = 0.25
Histology		
Adenocarcinoma (n = 197)	11.3 (10.2, 12.4)	570 (483, 657)
Squamous carcinoma (n = 74)	12.4 (11.3, 13.6)	629 (445, 813)
	P = 0.008	P = 0.75
Tumor location		
Upper esophagus (n = 13)	15.6 (11.4, 19.8)	973 (142,1804)
Mid esophagus (n = 50)	12.8 (11.0, 14.6)	425 (252, 598)
Lower esophagus (n = 136)	10.8 (9.6, 12.0)	586 (464, 708)
Junctional (n = 72)	11.6 (10.0, 13.1)	684 (430, 938)
	P = 0.010	P = 0.14

SUV_{max}: Maximum standardized uptake value.

Table 2 Influence of cancer stage on maximum standardized uptake value and survival

Factor	Mean SUV_{max} (95%CI)	Median survival in days (95%CI)
T stage		
T0 or T1 (n = 15)	3.1 (1.5, 4.7)	Not reached
T2 (n = 49)	8.7 (7.0, 10.4)	1225 (742, 1708)
T3 (n = 183)	12.7 (11.7, 13.7)	495 (413, 577)
T4 (n = 24)	14.1 (11.6, 16.7)	390 (186, 594)
	P < 0.001	P < 0.001
N stage		
N0 (n = 107)	9.1 (8.1, 10.2)	1094 (835, 1352)
N1 (n = 89)	12.9 (11.4, 14.5)	466 (371, 561)
N2 (n = 61)	13.4 (11.6, 15.1)	477 (307, 646)
N3 (n = 14)	14.4 (8.8, 19.9)	530 (350, 710)
	P < 0.001	P < 0.001
UICC stage		
Stage 0 or 1 (n = 45)	5.6 (4.2, 7.0)	2092 (1060, 3124)
Stage 2 (n = 50)	12.1 (10.6, 13.6)	780 (195,1365)
Stage 3 (n = 99)	11.9 (10.7, 13.2)	594 (473, 715)
Stage 4 (n = 77)	14.4 (12.6, 16.1)	349 (280, 418)
	P < 0.001	P < 0.001

SUV_{max}: Maximum standardized uptake value; UICC: Union for International Cancer Control.

when the two PET-CT scanners were employed. Specifically, with the study population divided into quintiles, there was no significant difference between successive quintiles of SUV_{max} (P = 0.55).

According to the multi-disciplinary panel, the treatment intention was radical (curative) for 182 patients and palliative for 89 patients. For the 182 patients treated with radical intent, principal treatment modality was surgery with or without neoadjuvant chemotherapy (114 patients), chemoradiotherapy (63 patients) and endoscopic mucosal resection (5 patients). Nineteen of the surgically

Table 3 Influence of treatment intent and modality on maximum standardized uptake value and survival

Factor	Mean SUV_{max} (95%CI)	Median survival in days (95%CI)
Treatment intention		
Curative (n = 182)	10.6 (9.7, 11.5)	984 (699, 1269)
Palliative (n = 89)	13.6 (11.9, 15.2)	370 (332, 408)
	P = 0.001	P < 0.001
Treatment type		
Endoscopic resection (n = 5)	1.3 (-1.0, 3.6)	Not reached
Surgical resection (n = 95)	10.7 (9.5, 11.9)	1285 (962, 1608)
Chemoradiotherapy (n = 63)	11.6 (10.0, 13.1)	700 (411, 988)
Palliative (n = 89)	13.8 (12.1, 15.5)	370 (349, 390)
Exploratory surgery (n = 19)	8.8 (6.6, 11.0)	340 (280, 400)
	P < 0.001	P < 0.001

SUV_{max}: Maximum standardized uptake value.

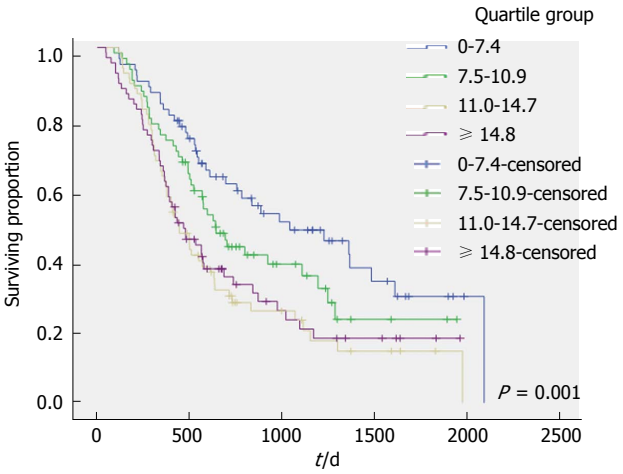

Figure 1 Survival of patients stratified into quartiles of maximum standardized uptake value.

treated patients underwent exploratory surgery because of the identification of unresectable T4 disease or peritoneal disease (19/114, 17%).

Analysis of SUV_{max} and survival

The outcome of univariate analysis comparing associations between patients factors (Table 1), tumor factors (Table 2) and treatment factors (Table 3) and SUV_{max} is shown. These show that SUV_{max} increased as disease burden (T stage, N stage and UICC stage) increased. Figure 1 plots survival for patients when stratified into quartiles of SUV_{max} (1st quartile 0-7.4, 2nd quartile 7.5-10.9, 3rd quartile 11.0-14.7, 4th quartile > 14.7). The strong link between SUV_{max} and survival is evident. The significance of SUV_{max} was lost on multivariate analysis. Using Cox regression analysis, the only factors significantly associated with survival were T-stage (P < 0.001) and UICC stage (P < 0.001). The same findings were evident when both the complete cohort was analyzed and when subgroup analy-

Table 4 Summary of literature reporting on prognostic value of maximum standardized uptake value in patients with esophageal carcinoma

Ref.	Patients (*n*)	Adeno-carcinoma (%)	Treatment intention of studied group	Median (or mean) SUV_{max}	SUV_{max} significant on univariate analysis	SUV_{max} significant on multivariate analysis	Other significant associations on multivariate analysis
Fukunaga *et al*[4], 1998	48	Not stated	Curative	7	Yes	Not assessed	Not assessed
Choi *et al*[5], 2004	69	0%	Curative	6.3/13.7 (thresholds)	Yes	No	UICC stage
Hong *et al*[6], 2005	47	87%	Curative	Not stated	No	No	Number of abnormalities on PET-CT
Stahl *et al*[7], 2005	40	100%	Curative	10.5	No	Not assessed	
van Westreenen *et al*[8], 2005	40	70%	Curative and palliative	6.7	Yes	No	Treatment
Cerfolio *et al*[9], 2006	89	53%	Curative	6.6	Yes	Yes	UICC stage
Choi *et al*[10], 2006	51	0%	Curative	Not stated	Yes	No	UICC stage, N1 status (on PET-CT), immunohistochemical markers
Westerterp *et al*[11], 2008	26	100%	Curative	0.26	Yes	Not assessed	
Omloo *et al*[12], 2008	125	85%	Curative	0.27	Yes	No	UICC stage
Cheze-Le Rest *et al*[13], 2008	47	77%	Curative	9	Yes	Yes	Treatment, number of abnormalities on PET-CT
Chatterton *et al*[14], 2008	129	19%	Curative and palliative	8.2	No	Not assessed	Not assessed
Makino *et al*[15], 2008	38	100%	Curative	11.1	Yes	No	N1 status (on PET-CT)
Javeri *et al*[16], 2009	161	100%	Curative	10.1	No	No	
Kato *et al*[17], 2009	184	0%	Curative	4.5	Yes	Yes	N1 status
Rizk *et al*[18], 2009	189	100%	Curative	4.5 (preset threshold)	Yes	Not assessed	Not assessed
Sepesi *et al*[19], 2009	72	83%	Curative	6.2	Yes	Yes	
Shenfine *et al*[20], 2009	45	100%	Curative	5.7	Yes	No	UICC stage
Hyun *et al*[21], 2010	151	3%	Curative and palliative	17.2	Yes	No	UICC stage, metabolic tumor volume
Brown *et al*[22], 2012	103	80%	Curative	6.4 (early)/8.8 (later scans)	Yes	No	N1 status, age
Gillies *et al*[23], 2012	121	100%	Curative	8.5	Yes	No	N1 status (on PET-CT)
Chan *et al*[24], 2013	185	75%	Curative and palliative	8.9	Yes	No	N1 status, tumor volume on EUS

SUV_{max}: Maximum standardized uptake value; UICC: Union for International Cancer Control; PET-CT: Positron emission tomography-computed tomography.

sis of individual treatment groups (chemoradiotherapy, surgery, palliative chemotherapy) and histologic subtype (adenocarcinoma, squamous carcinoma) was performed.

DISCUSSION

Twenty-one studies published to date have assessed the influence of pretreatment SUV_{max} on the prognosis of cancer of the esophagus in 1960 patients (Table 4)[4-24]. By cancer subtype, the proportion of patients with adenocarcinoma in the studies has ranged from 0% to 100%, with a median of 78%. As was noted in the current study, squamous carcinoma is associated with higher FDG uptake than adenocarcinoma. Sixteen of the studies assessed only patients being treated with radical intent, either surgery (with or without neoadjuvant chemotherapy) or chemoradiotherapy. Only four studies assessed patients treated with both radical and palliative intent. The current study represents the largest unselected study to date.

There were wide variations in the median, mean and threshold SUV_{max} noted in the published studies. The median value of 10.9 identified in the current study was higher than the majority of the studies and likely reflects the unselected population evaluated. Of note, the scans obtained in this study were obtained using two PET-CT machines, although there was no evidence that this had any influence on the measurements.

Pan *et al*[3], in a meta-analysis of the literature published up to 2009 identified SUV_{max} to be associated with a hazard ratio of 1.86 for overall survival, with higher values reflecting poorer survival. The authors however assessed the link between uptake and survival using univariate analysis. In the current study, a significant link between SUV_{max} and prognosis was noted on univariate analysis, but this effect disappeared on multivariate analysis. Table 4 indicates that 17 of the 21 studies (81%) identified a significant association between SUV_{max} and prognosis on univariate analysis, but only four of 16 studies (25%) found that this effect persisted on multivariate analysis.

The reason for this is likely to be the close relationship between SUVmax and UICC stage, and the overriding effect of UICC stage on all other prognostic markers. The literature taken en masse report similar themes.

Other factors that have been identified as being of prognostic value indirectly relate to cancer stage such as PET-CT N stage, the absolute number of abnormalities on PET-CT and the endoscopic ultrasound derived tumor node metastasis stage or tumor volume.

The current study has assessed the influence of a single pretreatment uptake value on cancer outcome, although other studies have suggested that serial PET-CT scanning may yield additional information by comparing pre- and post-treatment values[25,26]. At our institution, it is not standard practice to perform serial PET-CT. Patients undergo only one pretreatment examination.

We have previously shown that PET-CT alters the cancer stage in 26% of patients and that this translates into a change in management for 18%[2]. The implications of the current study are that the value of the PET-CT remains in the diagnosis of "occult" metastatic disease or confirming suspicious abnormalities on initial CT imaging. Its role is purely in triangulating with other information in order to predict pretreatment stage. The pretreatment SUV_{max} measurement, while closely linked to prognosis does not provide additional meaningful information that can be used in clinical decision making.

Several studies have noted that FDG uptake in regional lymph nodes may provide additional prognostic information[10,15,22-24]. At our institution, no attempt has been made to stage local peritumoral lymphadenopathy on the basis of PET-CT. We have considered the spatial resolution of the imaging insufficient to allow distinction between primary tumor and local lymphadenopathy. Local nodal staging is assessed by endoscopic ultrasound.

In conclusion, this study did not demonstrate the utility of PET-CT scanning, over and above determination of UICC stage. Pre-treatment SUV_{max} did not yield additional useful information.

COMMENTS

Background

Positron emission tomography-computed tomography (PET-CT) imaging is routinely employed in the staging of esophageal cancer. Its principal role is in the identification of metastatic disease. Some previous reports have suggested that the fluorodeoxyglucose (FDG) uptake [maximum standardized uptake value (SUV_{max})] may afford additional prognostic information.

Research frontiers

The research hotspot is to determine whether or not the effect of SUV_{max} on prognosis is independent of known prognostic markers, such as Union for International Cancer Control (UICC) stage

Innovations and breakthroughs

Univariate analysis identified that prognosis was linked to baseline pre-treatment) SUV_{max} in patients with esophageal cancer. However, this effect did not persist on regression analysis, with conventional prognostic markers (UICC stage and tumor stage) assuming significance.

Applications

The principal value of PET-CT in this patient group remains the identification of distant metastatic disease.

Peer review

The authors are to be congratulated on their effort. Although a retrospective study, it is well written and the authors have experience in this technology. They have addressed the clinical question about the independent effect of the prognostic information that maximum PET-CT FDG uptake could provide.

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REVIEW

Operable gastro-oesophageal junctional adenocarcinoma: Where to next?

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Abstract

Oesophageal junctional adenocarcinoma is a challenging and increasingly common disease. Optimisation of pre-operative staging and consolidation of surgery in large volume centres have improved outcomes, however the preferred adjunctive treatment approach remains a matter of debate. This review examines the benefits of neoadjuvant, peri-operative, and post-operative chemotherapy and chemoradiotherapy in this setting in an attempt to reach an evidence based conclusion. Recent findings relating to the molecular characterisation of oesophagogastric cancer and their impact on therapeutics are explored, in addition to the potential benefits of fluoro-deoxyglucose positron emission tomography (FDG-PET) directed therapy. Finally, efforts to decrease the incidence of junctional adenocarcinoma using early intervention in Barrett's oesophagus are discussed, including the roles of screening, endoscopic mucosal resection, ablative therapies and chemoprevention.

Key words: Oesophageal adenocarcinoma; Junctional adenocarcinoma; Gastric adenocarcinoma; Peri-operative chemotherapy; Pre-operative chemoradiotherapy; Molecular profiling; Fluoro-deoxyglucose-positron emission tomography; Barrett's oesophagus; Chemoprevention

Core tip: Cancer of the gastro-oesophageal junction is an increasingly common phenomenon. For patients with operable junctional cancer, the only curative treatment option is surgery, however the optimal peri-operative treatment is controversial. We review the evidence supporting the use of chemotherapy and chemoradiotherapy in the pre- and postoperative settings for these patients, and go on to highlight how current research into the molecular mechanisms underpinning gastro-oesophageal cancer may lead to future effective treatment options.

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INTRODUCTION

Adenocarcinoma of the oesophagogastric junction presents an increasingly common dilemma in many affluent countries, and the optimal treatment approach for patients with resectable disease is a matter of some controversy[1]. In addition to surgery for their cancer, and depending on geographical location and physician preference patients may undergo neoadjuvant, peri-operative, or post-operative chemotherapy, or pre- or post-operative chemoradiotherapy[2-4]. Unfortunately, despite improvements in staging and patient selection, long term survival following resection remains relatively poor and further refinement of treatment paradigms and novel therapeutic interventions are required. This aim of this review is to assess the current status of our knowledge on tumours of the gastroesophageal junction with respect to tumour

Table 1 Selected trials of peri-operative therapy for junctional oesophageal adenocarcinoma

Trial	Year	% Junctional adenocarcinoma or lower oesophageal tumours	*n*	Treatment	Survival (%)
Peri-operative chemotherapy					
MAGIC[5]	2006	Adenocarcinoma 100%	503	Surgery	23.00
		Lower oesophageal/GEJ 26%		Peri-operative chemotherapy	36.30 (5-year-OS)
FNCLCC-FFCD[6]	2011	Adenocarcinoma 100%	224	Surgery	24
		Lower oesophagus 11%, GEJ 64%		Peri-operative chemotherapy	38 (5-year-OS)
OEO2[7,8]	2009	Adenocarcinoma 66.5%	802	Surgery	17.10
		Lower 1/3 and cardia 75%		Neoadjuvant chemotherapy	23.00 (5-year-OS)
Pre-operative chemoradiotherapy					
Stahl[13]	2009	Adenocarcinoma 100%	126	Neoadjuvant chemotherapy	27.70
		GEJ 100%		Neoadjuvant chemoradiotherapy	47.40 (3-year-OS)
Tepper[14]	2009	Adenocarcinoma 75%	56	Surgery	1.79y
		Distal oesophagus/GEJ 100%		Neoadjuvant chemoradiotherapy	4.48y (median OS)
CROSS[15]	2012	Adenocarcinoma 74%	366	Surgery	44
		Distal 1/3 oesophagus 57, GEJ 24%		Neoadjuvant chemoradiotherapy	58 (3-year-OS)
Post-operative chemoradiotherapy					
INT-0116[16]	2001	Adenocarcinoma 100%	556	Surgery	41
		Cardia 20%		Adjuvant chemoradiotherapy	50 (3-year-OS)

DFS: Disease free survival; GEJ: Gastroesophageal junction; OS: Overall survival; SCC: Squamous cell carcinoma.

biology and therapy and to examine how developments in targeted therapy, radiotherapy, screening, and chemoprevention may improve outcomes for patients with this disease.

PERI-OPERATIVE CHEMOTHERAPY

In Western populations, many patients presenting with junctional adenocarcinoma have relatively locally advanced disease at presentation, and whilst there may be debate regarding the optimal treatment approach, there is agreement that something more than surgery is required to increase survival (Table 1). In Europe and selected United States academic centres, peri-operative chemotherapy is the treatment of choice for these patients. This choice is based on the United Kingdom MRC MAGIC trial, which treated over 500 patients with stomach, junctional or oesophageal tumours to either surgery alone or surgery plus peri-operative chemotherapy with epirubicin, cisplatin and 5-fluorouracil (5-FU)[5]. Peri-operative chemotherapy led to a 37% reduction in the risk of progression following surgical resection and improved 5 year survival from 23% in the surgery alone arm to 36% in those treated with chemotherapy (HR = 0.75, 95%CI: 0.60-0.93; $P = 0.009$). In MAGIC one quarter of patients had tumours of the gastroesophageal junction (GEJ) or lower oesophagus and subgroup analysis demonstrates that the greatest benefit was seen in patients with junctional tumours. These results are supported by the results of the randomised phase Ⅲ FNCLCC/FFCD French study in which 224 patients were randomised to surgery alone or peri-operative cisplatin and 5-fluorouracil chemotherapy[6]. The results from this study (in which 75% of patients had junctional tumours) are remarkably similar to those seen in MAGIC, with an improvement in 5 year overall survival from 24% to 38% (HR = 0.69, $P = 0.02$) for the interventional arm.

The aim of peri-operative chemotherapy is two-fold; firstly to downstage the primary tumour with a view to obtaining an R0 resection, and secondly to treat occult micro-metastatic disease. The neoadjuvant component of both MAGIC and the French study improved curative resection rates for patients in both these trials, in MAGIC 79.3% of chemotherapy patients were curatively resected compared to 70.3% in the surgery alone arm ($P = 0.03$), these figures are 84% and 73% respectively for the FFCD trial ($P = 0.04$). That subclinical micrometastases are eliminated is demonstrated by the almost uniform 35%-37% reduction in disease recurrence which seen across the two studies.

NEO-ADJUVANT CHEMOTHERAPY ALONE: IS IT ENOUGH?

Interestingly, a neo-adjuvant chemotherapy alone approach (with no post-operative component) does not appear to provide the same benefit to patients with oesophagogastric cancer. In the MRC OE02 study 802 patients with primarily oesophageal cancer (two thirds adenocarcinoma) were randomised to surgery alone or 2 cycles of cisplatin and 5-FU prior to surgery[7,8]. Although this study did demonstrate a survival benefit for patients treated with chemotherapy regardless of histology (5 year survival 23% *vs* 17%, $P = 0.03$), these results are not consistent with the results of the RTOG 8911 trial ($n = 467$) in which no difference was seen in the survival outcomes for a similar group patients treated with pre-operative chemotherapy[9]. Consistent with the negative results of the RTOG 8911 study are those of the smaller EORTC 40954 trial ($n = 144$, of whom half were junctional tumours). This study demonstrated an increase in the R0 resection rate following pre-operative cisplatin and 5-FU chemotherapy, but no improvement in overall survival[10]. These somewhat heterogeneous results have been combined in a meta-analysis which did demonstrate an improvement in survival for the neoadjuvant chemotherapy approach (HR = 0.90 for neoadjuvant chemotherapy, 95%CI: 0.81-1.00, $P = 0.05$)[11]. The benefit seen

appears to be due to the adenocarcinoma population (HR = 0.78, P = 0.014) as no significant difference was seen in the squamous cell carcinoma analysis. Therefore, although neoadjuvant chemotherapy alone for junctional tumours is not as clearly advantageous as treatment given both pre- and post-operatively, it is a reasonable choice if patients cannot tolerate post-operative chemotherapy.

NEOADJUVANT CHEMORADIOTHERAPY: DOES MAXIMISING LOCAL CONTROL LEAD TO IMPROVED SURVIVAL?

Response rates to radiotherapy are high, and if tumour downstaging in order to improve operative outcomes is the aim of therapy then radiotherapy has clearly defined benefits. However, if long term survival is the goal of treatment, many studies in junctional adenocarcinoma provide conflicting results. Analysis of the results of these studies must be careful, with consideration given to the external validity or generalizability of the data presented. Many trials present results based on both squamous cell carcinoma and adenocarcinoma patients between whom there are clear biological differences. Squamous cell carcinoma is exquisitely radiosensitive and may not require surgical resection if a pathological complete response is obtained following chemoradiotherapy. Adenocarcinoma is less likely to demonstrate such a response and will always require surgery in order to maximise the chance of long term survival. As such, caution must be used when extrapolating results from clinical trials as whole to biologically distinct patient groups.

Older studies of chemoradiotherapy for junctional cancers demonstrate mixed results. One of the first trials of neo-adjuvant cisplatin/5-FU based chemoradiotherapy for junctional type adenocarcinoma demonstrated a significant increase in survival for patients treated with combined modality therapy compared to those treated with surgery alone (16 m *vs* 11 m, P = 0.01)[12]. However, interpretation of these results should be made with care as this trial was small (n = 58), patients underwent limited staging by current standards (CXR and abdominal ultrasound only), and survival was poor in the control arm of the study. Following this two other small studies also demonstrated a benefit to this combined modality approach; the POET study randomised 126 patients with junctional adenocarcinoma to pre-operative chemotherapy and surgery or to induction chemotherapy followed by chemoradiotherapy and then surgery[13]. Survival was numerically improved by the addition of chemoradiotherapy (3 year survival 47% *vs* 28%, P = 0.07), but the study was underpowered due to low accrual and this did not reach statistical significance. CALGB 9781 (75% adenocarcinoma) also utilized a tri-modality approach in its experimental arm and demonstrated statistically superior survival for chemoradiotherapy when compared to surgery alone [Overall survival (OS) 4.5 years *vs* 1.8 years, P = 0.002], however the small number of patients in this trial (n = 56) and the lack of histological subgroup analysis limit interpretation of these interesting results[14].

The publication of the phase Ⅲ randomised CROSS trial which compared chemoradiotherapy (weekly carboplatin and paclitaxel with 41.4 Gy radiotherapy in 23 fractions over 5 wk) to surgery alone have lead to a paradigm shift in the treatment of junctional cancers in many institutions[15]. Three hundred and sixty six patients with oesophageal cancer (75% adenocarcinoma, 23% squamous cell carcinoma, 2% undifferentiated) were randomised, of whom the majority had tumours of the distal oesophagus (58%) or gastroesophageal junction (24%). Overall survival results for chemoradiotherapy in CROSS are compelling; survival was 24 mo for surgery alone compared to 49 mo for chemoradiotherapy (HR = 0.67, P = 0.003). However, several caveats apply. Firstly, the control arm in CROSS was surgery alone and the benefits of chemoradiotherapy compared to a contemporary control such as neoadjuvant chemotherapy are unknown. Secondly, in the adjusted survival analysis, the benefit of combination therapy is not significant for adenocarcinoma patients (P = 0.07), providing evidence that the overall results for the study were driven by the radiosensitivity of the squamous cell carcinoma patient population.

Chemoradiotherapy provides a clear advantage over chemotherapy alone in terms of pathological complete response and local recurrence. In CROSS 29% of patients overall demonstrated a complete response, however this was much more common in squamous cell cancers (49%) than in adenocarcinoma (23%). It is worth noting however, that although pathological complete response is an attractive endpoint, it is not necessary in order to achieve either tumour downstaging or an R0 resection, and that peri-operative chemotherapy alone can help to achieve both these endpoints as demonstrated in FNCLCC/FFCD and MAGIC[5,6]. Patients with junctional adenocarcinoma are also much more likely to harbour systemic micro-metastatic disease, and there is some concern that the systemic chemotherapy dose in CROSS is insufficient to eliminate these. This concern is highlighted by the fact that patients in CROSS with N1 or greater staging at presentation did not appear to benefit from chemoradiotherapy in the adjusted survival analysis (P = 0.21), implying that those at high risk of systemic relapse require a higher dose of systemic therapy in addition to an effective local treatment. Ultimately, there is no doubt that chemoradiotherapy is an excellent and frequently curative treatment for squamous cell carcinoma, and perhaps for very early node negative adenocarcinoma, but for patients with more locally advanced disease (who comprise the majority of patients seen), the evidence is less robust. A clinical trial comparing pre-operative chemoradiotherapy to peri-operative chemotherapy is underway (NCT01726452) and may in time give clarification to this important issue.

POST-OPERATIVE ADJUVANT CHEMORADIOTHERAPY

Post-operative adjuvant chemoradiotherapy is a strategy

more often adopted for resected gastric cancers in the United States[16]. In the landmark INT0116 study 556 patients were randomised to no treatment following surgery or to chemoradiotherapy consisting of 45 Gy with fluorouracil and leucovorin on a Mayo-type regimen schedule. A recently published 10 year follow up of this study demonstrated a long term survival benefit -50% of patients treated with chemoradiotherapy survived for five years, compared to 41% who received no further treatment with a 51% reduction in the risk of recurrence and a 32% reduction in the risk of death attributable to the interventional arm[17]. Although the majority (80%) of patients in the Intergroup study had true stomach cancers, approximately 20% had junctional adenocarcinoma, and for patients who have not undergone pre-operative treatment, this remains an evidence based treatment option. Of significant concern is the fact that most patients in this study did not have an adequate surgical resection (although this is more significant for gastric patients as opposed to oesophageal), and therefore radiotherapy in the post operative setting may merely compensate for insufficient surgery. A second problem with adjuvant chemoradiotherapy relates to tolerability; post-operative morbidity associated with gastrectomy is significant, and preoperative therapy tends to be much more tolerable to patients than post-operative. For example, in MAGIC and the FNCLCC/FFCD trials of peri-operative chemotherapy more than 85% of patients completed the neoadjuvant component of therapy, compared to less than 50% who complete the post-operative treatment[5,6]. Furthermore, as many patients with junctional adenocarcinoma have relatively bulky tumours which benefit from downstaging withholding therapy until the post-operative period may disadvantage the patient if attempting to achieve a curative R0 resection. Finally, although adjuvant chemotherapy alone as used in the ACTS-GC and CLASSIC studies provides a well defined survival benefit, these trials were almost completely composed of patients with resected gastric cancer, not junctional cancers, and also conducted in Asian populations with distinct surgical patterns and pharmacogenomic profiles[4,18]. For these reason, we prefer a pre-operative treatment approach for most patients with junctional adenocarcinoma if this is possible.

STRATEGIES TO IMPROVE OUTCOMES: NOVEL TARGETS, IMAGING AND EARLY INTERVENTION

Understanding disease biology leads to new targets for drug development

Despite the fact that oesophagogastric cancer is most prevalent in the affluent West and frequently in patients of higher socioeconomic status, survival remains mediocre. Although neoadjuvant or peri-operative therapy improves survival by over one third, relapse is common[5,6,15]. Interval improvement in outcomes have been due to stage migration which occurs as a result of improved staging, routine use of pre-operative positron emission tomography-computed tomography (PET-CT) and laparoscopy (in particular for patients with type Ⅲ tumours) may prevent futile surgery in up to one fifth of patients[19]. In order to build on these gains, it will be necessary to exploit the biology of the disease with changes in treatment approach to targeted drugs and/or immunotherapies, strategies which have yielded immense returns in other malignancies such as melanoma[20-22]. Although gastroesophageal cancer is currently treated as a single disease entity, this designation is based on anatomy, not biology and in future treatment paradigms may differ according to the underlying dysregulated molecular characteristics rather than the spatial location. From an epidemiological perspective, lower oesophageal and junctional cancers have a distinct set of risk factors, quite separate from distal gastric cancer. Whereas antral cancers are endemic in high risk areas, strongly correlated with *Helicobacter pylori* (*H. pylori*) infection, associated with poor diet and high salt intake, proximal cancers do not appear to be related to *H. pylori*, but are associated with obesity and chronic reflux oesophagitis[23-26]. Despite these differences, junctional and distal tumours both progress through a predictable path of histological changes en route to a Lauren's intestinal cancer phenotype and display similar biological behaviours. Ultimately junctional and distal cancers are more similar in nature to each other than to diffuse gastric cancer, a disease which when non-hereditary has no known epidemiological risk factors or precursor lesions, and which has a characteristic pattern of infiltrative peritoneal spread[27,28].

Molecular characterisation of gastric cancer has moved forward in recent years, with several groups attempting to define molecular signatures which may correlate with Lauren's pathological classification, provide information on prognosis or predict response to chemotherapy[29,30]. To date these approaches remain exploratory and require further validation in larger patient cohorts. Genome wide sequencing approaches have failed to identify many any significant driver mutations in oesophagogastric cancer; mutation rates in most well known oncogenes such as *BRAF*, *KRAS* and *PIK3CA* are relatively low and therefore it is difficult to determine whether they are associated with prognosis or response to chemotherapy[31,32]. Interestingly, in one study specifically exploring the genomic landscape of junctional adenocarcinoma almost half (49%) of recurrently mutated genes were unique to this tumour subsite when compared to previously reported mutations in gastric cancer[33]. Mutations are more frequent in key tumour suppressor genes such as *p53* and *ARID1A*, but unfortunately these are currently more difficult to exploit therapeutically, although potentially actionable activating mutations have also been documented in genes such as *FGFR4* and *HGF*[32,33]. Outside the spectrum of activating driver mutations, a significant proportion of gastroesophageal cancers demonstrate predominantly mutually exclusive amplification of receptor tyrosine kinases which may be targeted successfully with novel agents[34]. Over one third of cancers demonstrate amplification of one of *ERBB2*, *MET*, *FGFR*, *KRAS* or *EGFR*, and while it

appears that these cancers may be more clinically aggressive, they may also potentially benefit from treatment with novel targeted drugs[34-36].

Trastuzumab, the monoclonal antibody targeting the human epidermal growth factor receptor 2 (HER-2) receptor tyrosine kinase, was the first targeted therapy do demonstrate efficacy in oesophagogastric cancer, with an improvement in median overall survival to an unprecedented 16 mo for patients with advanced HER2 immunohistochemistry (IHC)3+ or IHC2+ fluorescence *in situ* hybridisation (FISH) positive tumours treated with chemotherapy plus trastuzumab[37]. This compares very favourably to median survival for similar patients treated with standard chemotherapy regimens which is generally less than one year[38,39]. In breast cancer, trastuzumab is associated with increased response rates and improved surgical outcomes when administered neoadjuvantly, and is curative in the adjuvant setting[40,41]. It is therefore a matter of regret that no registration study for trastuzumab was performed in conjunction with peri-operative chemotherapy for resectable gastroesophageal cancer, where up to 25% of patients with junctional cancers (who overexpress HER-2) could benefit[42]. However, for those who prefer a trimodality approach, a United States study will assess the benefits of the addition of trastuzumab to a CROSS like regimen of chemoradiotherapy for patients with resectable HER-2 positive oesophageal cancer (NCT01196390). The addition of pertuzumab (the monoclonal antibody inhibitor of HER-2 dimerization) to trastuzumab therapy has led to significant gains in overall survival for patients with metastatic breast cancer, as has the anti-HER2 antibody drug conjugate TDM1, and both pertuzumab (NCT01774786) and TDM1 (NCT01641939) are currently being evaluated in large, international randomised trials in HER2 positive gastric cancer in the first and second line setting respectively[43]. Therefore in future it is hoped it that these may play a role in the peri-operative setting.

Other potential pathways of interest for patients with gastroesophageal cancer include targeting angiogenesis, MET and fibroblast growth factor receptor (FGFR). Therapies targeting MET and FGFR, although promising from a preclinical perspective, have limited clinical evidence for efficacy at this stage beyond anecdotal reports from early phase clinical trials. However, there is substantial evidence to support an anti-angiogenic approach in operable gastroesophageal cancer. In a placebo controlled phase Ⅲ randomised trial the anti-VEGFR2 antibody ramicurumab led to a significant improvement in survival compared to best supportive care in previously treated advanced gastric cancer (OS 5.2 m *vs* 3.8 m HR = 0.78, $P = 0.047$)[44]. Interestingly, the benefit seen in terms of overall survival was comparable to that demonstrated in randomised studies of cytotoxic therapies in the same setting[45]. Ramicurumab has also improved survival when added to paclitaxel in the second line setting resulting in a median overall survival of an unprecedented 9.63 m for previously treated patients (HR = 0.807, 95%CI: 0.678-0.962; $P = 0.0169$)[46]. Furthermore, although in the phase Ⅲ randomised AVAGAST study for patients with advanced gastric cancer the addition of bevacizumab to cisplatin-fluoropyrimidine chemotherapy did not lead to a benefit in terms of overall survival, significant improvements in response rate and progression free survival were seen in the experimental arm[47]. As the goal of therapy in the peri-operative setting is to maximise response rate in order to achieve an R0 resection, then the addition of bevacizumab to peri-operative chemotherapy would appear to be a rational choice. This approach has been adopted in the large United Kingdom MRC ST03 trial, which will evaluate the addition of bevacizumab to peri-operative epirubicin, cisplatin and capecitabine chemotherapy (NCT00450203). This study completed recruitment of over one thousand patients in late 2013 and preliminary results are expected within the next two years.

IMAGE DIRECTED THERAPY: LARGER PATIENT COHORTS ARE NEEDED TO VALIDATE THIS PROMISING BIOMARKER

The routine use of PET-CT is helpful in staging patients with potentially operable junctional adenocarcinoma and may decrease the rate of futile surgery by identifying patients with CT-occult metastatic disease[19]. PET-CT has the potential to become a useful tool in assessing early response to treatment in oesophagogastric cancer, however studies evaluating this as a predictor of response have been small and lack validation. In the MUNICON I study of 54 patients with oesophageal cancer who failed to demonstrate a metabolic response following one cycle neoadjuvant chemotherapy (defined as ⩽ 35% decrease in SUV) no patient had a histological response and median survival for these patients was significantly worse than those who had a metabolic response (HR = 2.18, 95%CI: 1.32-3.62, $P = 0.002$)[48]. In the follow up MUNICON Ⅱ study patients who failed to demonstrate a metabolic (PET) response to a single cycle of pre-operative chemotherapy were treated with salvage chemoradiotherapy[49]. Although this did increase the pathological response rate compared to chemotherapy alone in the previous study it did not improve the R0 resection rate, and PET-non responders had almost half the rate of 2 year progression free survival of metabolic responders (64% for PET responders and 33% for PET non-responders (HR = 2.22, $P = 0.035$), highlighting the aggressive disease biology of non-responding patients. Unfortunately despite these intriguing findings the small number of patients in the MUNICON studies preclude these changing clinical practice and larger clinical trials will be required in order to do this; the CALGB group have initiated a study in which over two hundred patients with junctional adenocarcinoma are randomised induction chemotherapy with either FOLFOX (oxaliplatin plus fluorouracil) or carboplatin and paclitaxel with interval PET being performed following three cycles of treatment (NCT01333033). Patients who fail to respond on PET (⩽ 35% reduction in SUV) will cross over to the alternate treatment arm

of the study for concurrent chemoradiotherapy. The primary endpoint of this study is to increase the rate of pathological complete response in the initial PET non-responders to 20%, with progression free and overall survival being secondary endpoints. The UK MRC ST03 study (NCT00450203) which is evaluating the addition of bevacizumab to peri-operative chemotherapy is also performing a PET substudy which may provide further important information on this topic.

DECREASING CANCER RELATED MORTALITY WITH EARLY INTERVENTION

By the time symptoms such as dysphagia become apparent for patients with junctional adenocarcinoma the disease is often well established and frequently not amenable to surgery. Additionally, for those who are suitable for an operative approach the morbidity associated with such invasive surgery and peri-operative therapy is such that many patients may be excluded from curative treatment due to co-morbidity or performance status. However, for the small number of patients who are diagnosed with early stage cancers endoscopic resection may provide comparable results to surgical resection with less morbidity[50,51]. For patients with intramucosal carcinoma or high grade dysplasia with visible lesions endoscopic resection in a high volume centre is recommended with subsequent management dictated by the depth of tumour invasion on pathology[52]. Radiofrequency ablation is recommended for patients with early cancer or high grade dysplasia with no visible lesions/flat lining and for complete eradication of residual visible Barrett's oesophagus following endoscopic mucosal resection[51-55]. Based on randomised trial data, endoscopic resection of the entire Barrett's mucosa does not appear to provide any increased benefit over endoscopic resection of only visible lesions and radiofrequency ablation of the remainder of visible areas of Barrett's[56]. The case for endoscopic intervention is less clear for patients with low grade dysplasia, although there is clear evidence that ablative therapies can eradicate low grade dysplasia, given the low incidence of progression of such lesions to overt malignancy the benefit of this approach to patients is not definitively proved[52,57-60]. A randomised trial (SUrveillance *vs* RadioFrequency ablation - SURF) is currently addressing this issue[61].

Based on the non-operative interventions which are successful in treating Barrett's oesophagus it has been suggested that population screening for this condition could decrease oesophageal cancer related mortality. Although previously the rate of conversion was frequently estimated at approximately 0.5% annually the true rate is likely to be less than this[62,63]. Two recently published large population based studies containing almost twenty thousand patients between them estimate the risk to be between 0.12%-0.38% per annum[64,65]. If rates of conversion of Barrett's oesophagus to oesophageal adenocarcinoma are indeed this low, stratification of patients into high and low risk patient groups for screening will be necessary in order to maximise benefits to screened patients while optimising resource utilization. American Gastroenterological Association Guidelines suggest screening for Barrett's neoplasia only in persons with multiple risk factors such as chronic reflux, hiatus hernia, age $\geqslant$ 50, male sex, white race, elevated body mass index, and intra-abdominal body fat distribution, and British Society of Gastroenterology guidelines broadly concur with these, recommending surveillance in persons with at least of the above three risk factors, and also in those with a first degree relative with Barrett's oesophagus or oesophageal adenocarcinoma[52,66]. The recommendation to screen first degree relatives is based on research demonstrating that familial clustering of Barrett's oesophagus is not uncommon, with up to 28% first degree relatives of patients with oesophageal junctional adenocarcinoma or Barrett's with high grade dysplasia also demonstrating a Barrett's mucosa[67,68]. Recent gene wide association studies have confirmed this genetic propensity with Barrett's associated loci demonstrated in the MHC and on Ch16q24[69]. With respect to risk stratification of patients for consideration of endoscopy, there is some evidence that the frequency of symptoms of gastroesophageal reflux influences the risk of oesophageal adenocarcinoma ($\geqslant$ once per week symptoms odds ratio 4.9 $\geqslant$ daily symptoms odds ratio 7.4), however, as up to 40% of patients with oesophageal cancer have no history of reflux, focusing solely on symptomatic patients will have limited benefits with respect to mortality[70,71]. As the potential morbidity of endoscopic surveillance not insignificant, novel non-invasive techniques for screening for Barrett's have been developed. These include a capsule sponge (Cytosponge) where the patients ingests a gelatin capsule containing a mesh which is attached to a string, which is then withdrawn through the oesophagus collecting cells which are identified as Barrett's using an immunohistochemical marker[72]. In a prospective cohort study of 504 patients who had undergone 3 mo or more acid suppression therapy in the previous five years compared to the gold standard of endoscopic surveillance, the sensitivity and sensitivity of the Cytosponge were 73% and 94% for 1 cm or more circumferential length Barrett's and 90% and 94% for clinically relevant segments of 2 cm or more. However, given the low incidence of Barrett's in the population studied (3%), clearly improved patient selection for screening is required.

CHEMOPREVENTION

The effects of aspirin therapy on the risk of cancer occurrence have been demonstrated in the multiple observational studies; use of aspirin is associated with a significantly decreased risk of cancer death in patients both with and without pre-existing malignancies[73,74]. The prostaglandin pathway is dysregulated in the development of oesophageal cancer, as increased expression of cyclooxygenase 2 (COX-2) has been demonstrated in Barrett's oesophagus and inhibition of COX-2 activity leads

to growth inhibition of oesophageal cancer cell lines *in vitro*[75,76]. Inhibition of COX-1 (and modification of COX-2 activity) using high dose (≥ 325 mg/d) aspirin appears to decrease the risk of developing Barrett's oesophagus in a case control study (OR = 0.36; $P = 0.001$), and a meta-analysis of multiple cohort studies confirms that aspirin (OR = 0.64, 95%CI: 0.52-0.79) or other NSAID (HR = 0.65, 95%CI: 0.50-0.85) use is associated with a lower risk of oesophageal adenocarcinoma[77,78]. The large UK ASPECT trial (NCT00357682) has recruited over 2500 patients with Barrett's oesophagus and randomised these to aspirin plus acid suppression therapy *vs* acid suppression therapy alone; the results of this study are eagerly awaited. A further large randomised worldwide study (Add-Aspirin) will begin recruitment in 2014 to assess whether aspirin given following surgical resection of oesophageal cancer will decrease the risk of recurrent disease. Although the epidemiological evidence for risk reduction due to aspirin is compelling, due to the lack of randomised data available, the potential toxicity associated with aspirin use, and potential biases of the current data, neither the American Gastroenterological Association nor the British Society of Gastroenterology recommend routine use of aspirin as a chemopreventative measure for decreasing the risk of Barrett's or oesophageal adenocarcinoma, although screening patients for cardiovascular risk factors for which aspirin therapy may be indicated is warranted[52,67].

CONCLUSION

Junctional adenocarcinoma is a challenging disease. The rate of its rapid increase in prevalence does not appear to have peaked, and if levels of obesity also continue to escalate worldwide it is likely to become a significant global health issue. Although precursor lesions exist which are amenable to curative therapy, identification of at risk patients who would benefit from screening is currently difficult. Once an invasive cancer is established it is clear that for most patients further therapy in addition to surgery will help improve survival. Whether this is peri-operative chemotherapy or neoadjuvant chemoradiotherapy is a matter of contention. This question has been difficult to answer in a straightforward manner due to the design of previous clinical trials, where patients with junctional adenocarcinoma have been treated alongside patients with squamous cell carcinoma of the proximal oesophagus or distal gastric cancers. For the purpose of clarity we believe that any future trials should not include squamous cell cancers, which have an entirely different disease biology, and if including distal gastric cancers are powered for a relevant subset analysis. Exploitation of the underlying molecular aberrations seen in oesophagogastric cancer, in particular amplification of receptor tyrosine kinases may lead to significant improvements in survival - however use of these agents is at this time predominantly limited to the metastatic setting. Increased uptake of PET directed therapy may allow superior selection of patients for intensified pre-operative regimens or immediate resection in the absence of response and this widely available biomarker is currently underutilised. Finally, it is hoped developments in the field of chemoprevention using the widely available and inexpensive medications such as aspirin may decrease the risk of progression of Barrett's oesophagus to overt malignancy at low cost and toxicity.

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REVIEW

Primary tumor resection in colorectal cancer with unresectable synchronous metastases: A review

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Abstract

At the time of diagnosis, 25% of patients with colorectal cancer (CRC) present with synchronous metastases, which are unresectable in the majority of patients. Whether primary tumor resection (PTR) followed by chemotherapy or immediate chemotherapy without PTR is the best therapeutic option in patients with asymptomatic CRC and unresectable metastases is a major issue, although unanswered to date. The aim of this study was to review all published data on whether PTR should be performed in patients with CRC and unresectable synchronous metastases. All aspects of the management of CRC were taken into account, especially prognostic factors in patients with CRC and unresectable metastases. The impact of PTR on survival and quality of life were reviewed, in addition to the characteristics of patients that could benefit from PTR and the possible underlying mechanisms. The risks of both approaches are reported. As no randomized study has been performed to date, we finally discussed how a therapeutic strategy's trial should be designed to provide answer to this issue.

Key words: Colorectal cancer; Colorectal surgery; Chemotherapy; Colorectal primary tumor; Survival; Liver metastases

Core tip: The present review aimed to analyze all published data on whether primary tumor resection should be performed before chemotherapy administration in patients with colorectal cancer and unresectable synchronous metastases.

de Mestier L, Manceau G, Neuzillet C, Bachet JB, Spano JP, Kianmanesh R, Vaillant JC, Bouché O, Hannoun L, Karoui M. Primary tumor resection in colorectal cancer with unresectable synchronous metastases: A review. *World J Gastrointest Oncol* 2014; 6(6): 156-169 Available from: URL: http://www.wjgnet.com/1948-5204/full/v6/i6/156.htm DOI: http://dx.doi.org/10.4251/

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INTRODUCTION

With nearly 150000 new cases in the United States annually (about 1 million in developed countries) and 55000 annual deaths (about 500000 in developed countries), colorectal cancer (CRC) stands as the second leading cause of cancer death in Western countries and a significant public health issue[1]. In approximately 20% of patients, distant metastases are already present at the time of diagnosis[2]. The liver is the most common metastatic site. Surgery plays an important role in the treatment of patients with limited metastatic disease with 20%-50% rates of cure and long-term survival after complete R0 resection[3]. However, for the majority (75%-90%) of these CRC patients with synchronous liver metastases (SLM), there are no curative options, but a significant benefit in median overall survival (OS) and quality of life can be achieved with palliative systemic treatment, namely effective chemotherapy regimens and targeted biotherapies[4,5].

Patients with CRC and unresectable SLM may present with a variable degree of symptoms of their primary tumor. The indication of palliative primary tumor resection (PTR) prior to the initiation of systemic treatment is obvious in patients with primary tumor-related symptoms or complications (obstruction, bleeding, or perforation). However, in asymptomatic CRC patients with unresectable SLM, the indication of PTR as initial management remains questionable and its effect on survival and quality of life is uncertain. No randomized trial has answered to these questions to date[6-13].

Historically, many surgeons have advocated PTR, mainly to avoid potential related complications such as bleeding, perforation or obstruction and because it allows precise tumor staging[14,15]. However, during the past decade, several highly active systemic agents have become available for the treatment of metastatic CRC patients. These agents have increased the median survival duration from 9 to 12 mo with 5-fluorouracil alone, to 24 mo with the addition of modern cytotoxic and targeted agents[16-20]. Owing to the increased efficacy of chemotherapy on metastatic CRC as well as on primary tumor[21], complications from unresected primary tumor have become relatively infrequent. Therefore, there is a tendency among surgeons not to perform PTR in case of unresectable metastases. The possible influence of PTR on survival of patients with CRC and unresectable SLM has never been assessed properly. It has been suggested that PTR, in the setting of unresectable metastatic disease, was related to prolonged survival on multivariate analysis in the majority of these series[6-10,12,13,22]. Nevertheless, most studies reporting an association between PTR and prolonged survival have been limited by numerous selection biases. In addition, whether these two strategies impact patient's quality of life has never been evaluated. Finally, the relative low post-operative morbidity rates reported after laparoscopic resection in stage Ⅳ CRC[23-25] and the progress in perioperative management of these patients, have reinforced the debate between the two strategies (PTR *vs* no PTR). While waiting for a randomized study, the objective of the present work was to review the state of the art on the management of CRC patients with unresectable synchronous metastases, with particular focus on PTR.

TREATMENT OF METASTATIC COLORECTAL CANCER

When metastases of CRC patients are restricted to the liver, possible curative treatment can be obtained by surgical resection of the metastases. Patients with oligometastases restricted to the lungs may also be candidates for surgical resection. Complete surgical resection of metastatic lesions substantially improves overall survival rates to around 35%-60% in selected patients[3]. Even extra-hepatic disease is no longer a contraindication for surgery in selected patients[26]. Hyperthermic intraperitoneal chemotherapy is a promising treatment in selected patients with limited peritoneal carcinomatosis and long term survival can be achieved[27]. In all other cases, CRC patients with unresectable metastases are treated with systemic combination chemotherapy regimens. Most common combinations are oxaliplatin or irinotecan in addition to a fluoropyrimidine (capecitabine or 5-fluorouracil). Since the last decade, targeted biotherapies have been possibly administered in addition, such as antiangiogenic therapy (*i.e.*, bevacizumab) and anti-epidermal growth factor receptor antibodies *(i.e.*, panitumumab and cetuximab) in the setting of *KRAS* wild-type tumors. These systemic chemotherapeutic combinations have raised response rates to 40%-75% resulting in a median overall survival rate of approximately 24 mo[5,19,28,29]. With current chemotherapy regimens, around 20% of the tumors initially judged unresectable have been converted to resectable, leading to secondary curative surgery and similar prognosis than in patients who underwent surgery for initially resectable liver metastases[3,30].

IMPACT OF PRIMARY TUMOR RESECTION ON THE SURVIVAL OF PATIENTS WITH COLORECTAL CANCER AND UNRESECTABLE SYNCHRONOUS LIVER METASTASES

In patients with asymptomatic primary tumor and unresectable SLM, PTR prior to the initiation of systemic treatment is questioned. Its effects on survival and quality of life are uncertain[6-18,31,32]. No randomized control trial has been conducted to date.

Several studies have been performed to analyze the survival in patients with unresectable stage Ⅳ CRC un-

Table 1 Median survival (mo) in patients with unresectable metastatic colorectal cancer, according to whether primary tumor resection was performed or not

Ref.	Study period	Resection/ No resection	No.of patients	OS (mo)	*P* value
Scoggins *et al*[82]	1985-1997	Resection	66	14.5	0.59
		No resection	23	16.6	
Tebbutt *et al*[34]	1990-1999	Resection	280	14	0.08
		No resection	82	8.2	
Ruo *et al*[44]	1996-1999	Resection	127	16	< 0.001
		No resection	103	9	
Michel *et al*[90]	1996-1999	Resection	31	21	0.718
		No resection	23	14	
Law *et al*[35]	1996-1999	Resection	150	7	< 0.001
		No resection	30	3	
Benoist *et al*[79]	1997-2002	Resection	32	23	NS
		No resection	27	22	
Stelzner *et al*[45]	1995-2001	Resection	128	11.4	< 0.0001
		No resection	58	4.6	
Konyalian *et al*[36]	1991-2002	Resection	62	13	< 0.0001
		No resection	47	5	
Costi *et al*[91]	1994-2003	Resection	83	9	< 0.001
		No resection	47	4	
Yun *et al*[37]	1994-2004	Resection	283	15.3	< 0.001
		No resection	93	5.3	
Kaufman *et al*[92]	1998-2003	Resection	115	22	< 0.0001
		No resection	69	3	
Galizia *et al*[38]	1995-2005	Resection	42	15.2	0.03
		No resection	23	12.3	
Evans *et al*[70]	1999-2006	Resection	45	11	< 0.0001
		No resection	57	2	
Bajwa *et al*[39]	1999-2005	Resection	32	14	0.005
		No resection	35	6	
Mik *et al*[40]	1996-2000	Resection	52	21	NS
		No resection	82	14	
Frago *et al*[93]	2004-2008	Resection	12	23.7	0.008
		No resection	43	4.4	
Aslam *et al*[41]	1998-2007	Resection	366	14.5	< 0.005
		No resection	281	5.83	
Chan *et al*[11]	2000-2002	Resection	286	14	< 0.001
		No resection	125	6	
Seo *et al*[94]	2001-2008	Resection	114	22	0.076
		No resection	83	14	
Karoui *et al*[33]	1998-2007	Resection	128	30.7	0.031
		No resection	85	21.9	
Ferrand *et al*[22]	1997-2001	Resection	156	16.3	< 0.0001
		No resection	60	9.5	

OS: Overall survival.

dergoing PTR, in comparison with those who did not (Table 1). All were non-randomized and most were single-center and retrospective. In addition, the major drawback of these studies is that patients with a better World Health Organization performance status (WHO-PS) and better prognosis at baseline (less metastatic sites involved) were more likely to undergo surgery. Conversely, patients with extensive disease were more likely to be offered chemotherapy rather than surgery thus standing as a major selection bias. Similarly, only patients with good WHO-PS were able to tolerate a complete course of potentially toxic chemotherapeutic agents such as irinotecan and oxaliplatin. Another limitation is that reported data on the use of systemic therapy are scarce, which hardens the assessment of the influence of PTR on outcome. Despite these limitations, the median OS was improved in resected patients in the vast majority of studies.

Our group recently reported a 10-year retrospective experience of the management of metastatic colonic cancer in chemotherapy-eligible patients, managed in 6 Parisian university hospitals[33]. The primary aim of this study was to compare outcomes, including survival, in 208 patients with unresectable distant metastases undergoing either PTR (n = 85) or systemic chemotherapy (n = 123) as their initial treatment. Most patients had not received targeted therapy as first-line treatment. Median OS was nearly 9 mo longer after PTR than after initial systemic chemotherapy (30.7 mo *vs* 21.9 mo, adjusted HR= 0.56; P = 0.031). In this series, the 2 groups were different with respect to baseline carcinoembryonic antigen (CEA) level, which was lower in the colectomy group (P = 0.008), suggesting a lower disease burden[33]. Despite similar rates of chemotherapy administration, the secondary curative resection rate was higher in the PTR group than in patients treated with initial chemotherapy (32.9% *vs* 20.3%; P = 0.04), suggesting a lower metastatic burden and other potential unmeasured differences contributing to a greater response to chemotherapy. In an effort to take into account these differences, a propensity score was performed and used for adjustment. On multivariate analysis, first-intent PTR, secondary curative resection, well-differentiated primary tumor, liver-only metastases and addition of targeted therapy were independently associated with survival. After adjusting on the propensity score quartiles, as well as for the quantitative value of this score, these five factors were still independently associated with survival[33].

A recent meta-analysis of 8 retrospective comparative studies including 1062 patients has reported an improvement in the survival of those with palliative PTR, with an estimated median gain of 6 mo (standardized HR = 0.55; 95%CI: 0.29-0.82; P < 0.001)[8]. The initial heterogeneity between the studies was amended after excluding one study[34], in which survival was not the primary endpoint. The authors also reported that PTR was not associated with increased secondary resectability of metastases following chemotherapy, in comparison with patients treated with chemotherapy alone (HR = 0.85; 95%CI: 0.4-1.8, P = 0.66)[8].

Venderbosch *et al*[12] performed a retrospective analysis of two phase Ⅲ studies (CAIRO and CAIRO2), investigating the prognostic and predictive value of PTR in patients with synchronous stage Ⅳ CRC treated with systemic therapy. In the CAIRO study, 258 patients underwent PTR (*vs* 141 who did not) and showed increased median OS (16.7 mo *vs* 11.4 mo, respectively; HR = 0.61; P < 0.0001) and progression-free survival (PFS) (6.7 mo *vs* 5.9 mo, respectively; HR = 0.74; P = 0.004). Similarly, in the CAIRO2 study, 289 patients underwent PTR (*vs* 159 who did not) and showed increased median OS (20.7 *vs* 13.4 mo; HR = 0.65; P < 0.0001) and PFS (10.5 mo *vs* 7.8 mo; HR = 0.78; P = 0.014)[12]. A major limitation of these results consisted in the fact that the decision of PTR was made prior to study inclusion. Besides, no information about the reasons for non-resection were provided, such as absence of symptoms, unresectability of the primary

Table 2 Prognostic factors associated with overall survival in patients with unresectable metastatic colorectal cancer, according to whether primary tumor resection was performed or not

Ref.	Resection/No resection	No. of patients	OS (mo)	*P* value	PTR on multivariate analysis [95%CI]	Other independent prognostic factors
Tebbutt *et al*[34]	Resection	280	14	0.08	No	WHO-PS < 2, no peritoneal dissemination, low phosphatase alkaline and serum albumin levels
	No resection	82	8.2			
Law *et al*[35]	Resection	150	7	< 0.001	OR = 0.42 (0.27–0.66)[1]	Unilobar LM involvement, no ascites, no chemotherapy
	No resection	30	3		*P* < 0.001	
Stelzner *et al*[45]	Resection	128	11.4	< 0.0001	HR = 0.50 (0.27–0.90)	No chemotherapy, ASA score < 3, WHO-PS < 2, CEA level, age < 75 yr, extent of metastases, extent of primary tumor
	No resection	58	4.6		*P* = 0.021[2]	
Konyalian *et al*[36]	Resection	62	13	< 0.0001	HR = 0.3 (0.2–0.6)	Liver involvement < 50%
	No resection	47	5		*P* < 0.0001[3]	
Yun *et al*[37]	Resection	283	15.3	< 0.001	HR = 0.53 (0.38–0.73)	Metastatic site ⩽ 1, high CEA level, chemotherapy, well-differentiated primary tumor
	No resection	93	5.3		*P* < 0.001	
Galizia *et al*[38]	Resection	42	15.2	0.03	OR = 3.91 (2.83-4.99)	WHO-PS < 2, liver involvement < 50%
	No resection	23	12.3		0.26 (0.20-0.35)[1] *P* = 0.001	
Bajwa *et al*[39]	Resection	32	14	0.005	OR = 0.26 (0.13-0.52)	Left sided primary tumor, unique primary tumor
	No resection	35	6		*P* = 0.0001	
Mik *et al*[40]	Resection	52	21	NS	HR = 0.58 (0.36–0.82)[1]	Unilobar LM involvement
	No resection	82	14		*P* = 0.004	
Aslam *et al*[41]	Resection	366	14.5	< 0.005	*P* < 0.001	Age < 80 yr, non-locally advanced primary tumor, N + stage
	No resection	281	5.83			
Karoui *et al*[33]	Resection	128	30.7	0.031	HR = 0.56 (0.38–0.83)[1]	Secondary curative resection, well-differentiated primary tumor, anti-VEGF treatment, no extra-hepatic metastases
	No resection	85	21.9	-	*P* = 0.004	
Platell *et al*[83]	Resection	243	-		HR = 0.51 (0.37–0.69)	Chemotherapy, radiotherapy, ASA score < 3
	No resection	70	-		*P* = 0.0001	
Venderbosch *et al*[12]	Resection	286	14	< 0.001	HR = 0.73 (0.58-0.93)	-
	No resection	125	6		*P* = 0.01	
Ferrand *et al*[22]	Resection	156	16.3	< 0.0001	HR = 0.42 (0.30-0.60)	WHO-PS < 2, distal colon or rectal primary tumor, one metastatic site and alkaline phosphatase ⩽ 300 UI/L
					P < 0.0001	
	No resection	60	9.5			

[1]For readability of the Table, some ORs and HRs have been recalculated with " No resection" as reference for the multivariate analysis of survival; [2]excluding postoperative mortality and complicated primary tumor; [3]PTR was independently associated with increased survival probability, while adjusting on patient's age, sex and degree of hepatic tumor involvement. OS: Overall survival; PTR: Primary tumor resection; OR: Odds ratio; HR: Hazard ratio; LM: Liver metastases; ASA: American society of anesthesiology; CEA: Carcinoembryonic antigen; VEGF: Vascular-endothelial growth factor; WHO-PS: World health organization performance status; NS: Not significant.

tumor, poor patient condition and/or symptomatic metastases requiring rapid initiation of systemic treatment. Obviously, many differences were likely to stand between patients undergoing PTR or not. However, on multivariate analysis, PTR remained a significant prognostic factor in the CAIRO2 study and in the subgroup of patients with one metastatic site in the CAIRO study[12].

Finaly, Ferrand *et al*[22] recently performed an analysis of 260 patients included in the Fédération Francophone de Cancérologie Digestive 9601 phase Ⅲ trial, which compared different first-line single-agent chemotherapy regimens in patients with stage Ⅳ CRC. Two-year OS and 6-mo PFS were significantly better in the resection group than in the non-resection group (24% *vs* 10%; *P* < 0.0001 and 38% *vs* 22%; *P* = 0.001, respectively). The gain of OS was 6.8 mo. These results remained significant even after exclusion of the 49 patients with rectal cancer. In multivariate analysis, PTR was the most significant prognostic factor (HR = 0.42; 95%CI: 0.30-0.60, *P* < 0.0001). In this study, 4 factors were associated with a decreased survival: poor WHO-PS, multiple metastatic sites, proximal colonic primary tumor and high baseline alkaline phosphatase level.

WHICH PATIENTS WITH COLORECTAL CANCER AND UNRESECTABLE SYNCHRONOUS LIVER METASTASES ARE LIKELY TO BENEFIT FROM PRIMARY TUMOR RESECTION?

Some comparative studies conducted multivariate analysis to determine which clinical, tumor and therapy variables were associated with survival between patients managed by primary surgery or immediate chemotherapy[10] (Table 2). In addition to PTR, several factors were found to have independent prognostic influence: age, American society of anesthesiology (ASA) score, WHO-PS, preoperative CEA levels, primary tumor location, size and differentiation, extent of metastatic liver spread, peritoneal dissemination and extra-hepatic metastases. Other independent factors have been less frequently reported, such as serum albumin, alkaline phosphatase levels, lymph node involvement, ascites, number of metastatic sites and the administration of targeted therapy. Some works also emphasized that tumor burden (primary tumor and/or metastatic

Table 3 Prognostic factors after primary tumor resection on multivariate analyzes

Ref.	Metastatic spread	No. of patients	Prognostic factors or predictive factors of postoperative morbimortality
Rosen *et al*[43]	Liver, Peritoneum	125	Age < 65 yr, limited LM, no peritoneal carcinomatosis
Ruo *et al*[44]	Liver, peritoneum, retroperitoneal lymph nodes, lung, bone, brain	123	Liver involvement < 25%
Stelzner *et al*[45]	Mainly liver	186	WHO-PS, ASA grade, low CEA level, metastatic load, chemotherapy
Vibert *et al*[47]	Liver	80	Serum AST level < 50 IU/l, age < 75 yr
Yun *et al*[37]	Liver, peritoneum, lung	503	CEA level, well-differentiated primary tumor, chemotherapy
Kleespies *et al*[46]	Mainly liver, lung, peritoneum	233	Liver involvement < 50%, chemotherapy, pT4 and/or N+ stage
Costi *et al*[48]	Mainly liver, peritoneum	71	Age < 80 yr, nodal stage
Stillwell *et al*[31]	Liver and extra-hepatic	379	Nodal stage < N2, well-differentiated primary tumor, no postoperative complications, no apical lymph-node

LM: Liver metastases; ASA: American society of anesthesiology; CEA: Carcinoembryonic antigen; WHO-PS: World health organization performance status.

disease) was significantly related to survival[22,33-42]. Bilobar liver metastases were associated with decreased survival compared to unilobar location, the risk of cancer-related death being five-fold increase in case of > 50% liver involvement[35,36,38,40]. Similarly, peritoneal and omental metastases are significantly related to poorer survival[34].

Furthermore, several studies reported multivariate analysis of predictive factors affecting outcome after PTR in patients with CRC and unresectable SLM. The main factors influencing outcome were the extent of liver disease[42-46], age[43,47,48] and tumor differentiation[31,37] (Table 3).

The results of the study by Vibert *et al*[47] suggested that patients older than 70 years with elevated aspartate aminotransferase enzymes may not benefit from palliative PTR and could be offered chemotherapy if suitable. A retrospective review of 503 palliative PTR found that predictors of survival included serum CEA level, degree of differentiation of the tumor, successful PTR and the use of chemotherapy[37]. In another study, age > 65, the presence of carcinomatosis and extensive bilobar liver involvement were not only associated with decreased survival after PTR, but with increased morbidity and mortality as well[43]. Kuo *et al*[49] suggested that patients older than 65 with multiple-site metastases, intestinal obstruction, preoperative CEA levels > 500 ng/mL, lactate dehydrogenase > 350 units/L, hemoglobin < 10 g/dL, or liver tumor burden > 25% exhibited worse survival following surgery than those without.

To summarize, most of studies suggested that liver burden > 50% and extra-hepatic metastatic disease (peritoneal carcinomatosis, lung metastases) were poor prognostic factors in patients with CRC and unresectable SLM, as well as advanced age and poor WHO-PS. Interestingly, this appears to have remained unchanged with time despite the advances in the surgery and systemic therapy. Thus, patient selection is a critical issue, and the decision for PTR should take into account these prognostic factors.

UNDERLYING HYPOTHESES FOR INCREASED SURVIVAL IN PATIENTS UNDERGOING PTR

Reasons why PTR is associated with better outcomes in patients with CRC and unresectable metastases are still unclear. The improvement in survival following PTR may be attributed to a better response to chemotherapy after reduction of tumor burden. This has been demonstrated by the proven benefit of resecting primary renal and ovarian tumors in the presence of metastatic disease[50,51]. Survival of resected patients might also be improved because they are less likely to develop obstruction and perforation, complications known to carry heavy operative mortality and morbidity[8]. Besides, surgical removal of primary tumor may restore immunocompetence, even at a metastatic stage, as shown in a murine model xenografted with 4T1 mammary carcinoma[52].

It has been suggested that the interaction between primary tumor and target organs of metastasis dictates the progression from micro- to macrometastases[53]. Indeed, the primary tumor may induce, in these distant organs, a prosperous environment to enhance the growth of metastatic deposit (seed and soil theory). Vascular endothelial growth factor receptor 2 (VEGFR-2) expressing circulating tumor cells settle in the pre-metastatic niches, previously colonized by hematopoietic cells expressing VEGFR-1[54]. The recent study by van der Wal *et al*[55] suggested that PTR could prevent the liver parenchyma from soiling from micrometastases. Indeed, the authors demonstrated that the expression levels of angiogenetic markers (CD31, VEGF-A, VEGFR-1, VEGFR-2, Placental Growth Factor, Hypoxia-induced Factor 1 alpha, Angiopoietin-2 and its receptor Tie-2, all assessed using reverse transcription-polymerase chain reaction) were higher in the liver parenchyma adjacent to metastases, both in patients with simultaneous resection of both their primary tumor and liver metastases, and in those who underwent metastases removal several months after PTR. Moreover, the simultaneous resection group showed the highest Ang-2/Ang-1 (proangiogenic) ratio both in the metastases and the adjacent liver. These results suggested that in the presence of the primary tumor, the liver parenchyma adjacent to metastases provided an angiogenic prosperous soil for metastatic tumor growth and may explain the association of PTR with improved survival[55]. These results are also in concordance with the prognostic role of anti-VEGF based treatment we found on multivariate analysis in our series[33].

In contrast, several studies based on PET-scan and histology showed an increased growth of liver metastases following PTR, as determined by an increased vascular density, proliferation rate, and metabolic growth rate[56-59]. These data suggest that the outgrowth of metastatic disease may, at least partly, be downregulated by the primary tumor, notably by inhibiting metastatic angiogenesis. In mouse models, pulmonary metastases showed rapid progression after PTR, which was considered to be the result of depletion of the antiangiogenic compound angiostatin produced by the primary tumor[53,56,60]. After PTR, antiangiogenic effects disappear, and metastases undergo an "angiogenic switch", leading to angiogenesis and enhanced tumor growth[60]. In addition, major surgery induces a transient immunodepression which may promote tumor growth[61,62]. Romano *et al*[63] reported that 29% of CRC patients had lymphocytopenia at baseline. In comparison, 14 d after surgery, values below normal range for total lymphocyte count and helper T-cells were found in 44% and 53% of cases, respectively. Recovery of postoperative surgery-related lymphocytopenia occurred late only in patients with normal count at baseline. In a rat model, perioperative restoration of lymphocyte proliferation levels either by levamisole or maleic anhydride-divinyl ether-2 resulted in fewer hepatic metastases, suggesting the critical role of immunomodulation in the development of metastases[64,65]. Notably, perioperative blood transfusions have been shown to exert an immunosuppressive effect on patients with CRC and are independently associated with a poor prognosis[66,67].

However, these pro-tumoral effects seem to be counterbalanced by previously described anti-tumoral effects of PTR, as most studies have reported an association between PTR an improved outcome. Overall, it seems ethically relevant to perform a clinical trial comparing PTR to conservative strategy, as data remains controversial regarding PTR consequences on tumor evolution. Indeed, influence of primary tumor on angiogenesis of metastases are based on experimental studies, which does not necessarily translate clinically into a modification of patient survival. Studies that showed an advantage of PTR had such selection bias that interpretation of their findings are difficult, even with the use of multivariate analyzes or propensity scores. Definitive response regarding the interest of PTR in stage Ⅳ CRC patients could only be obtained with a randomized trial with selective inclusion criteria and comparable arms.

IMPACT OF PRIMARY TUMOR RESECTION ON QUALITY OF LIFE OF PATIENTS WITH COLORECTAL CANCER AND UNRESECTABLE SYNCHRONOUS LIVER METASTASES

The effect of PTR and chemotherapy on quality of life has never been specifically evaluated. In the palliative care setting, determining the effect of PTR on quality of life would help clinicians and patients deciding the most adapted primary strategy. Primary-related symptoms or complications, postoperative morbidity following PTR (either electively or for complications), total length of hospital stay and tolerability of chemotherapy (according to the presence or absence of the primary tumor) may all contribute to impact quality of life. They should thus stand as secondary endpoints in a future prospective randomized study evaluating the impact of PTR in CRC patients with unresectable synchronous metastases. Quality of life could be assessed in both arms with the use of validated questionnaires such as the european organization for research and treatment of cancer quality of life questionnaire core 30 (EORTC QLQ-C30) and EORTC-CR29, at baseline and after initiation of treatment (surgery or chemotherapy) with longitudinal follow-up.

WHAT ARE THE RISKS OF UNRESECTED PRIMARY TUMOR-RELATED COMPLICATIONS UNDER CHEMOTHERAPY?

PTR has been traditionally advocated in the setting of metastatic CRC, to prevent symptoms and complications linked to primary tumor, such as obstruction, perforation or bleeding. Emergency surgery is associated with high morbidity and even mortality[45,68-70]. The risk of local complications related to tumor left in situ, during initial chemotherapy, varied from 8.5% to 30% and was dominated by the risk of obstruction (6%-29%) (Table 4). These results require cautious interpretation, as they came from old retrospective series that involved few patients supported for long periods with heterogeneous chemotherapy regimens. In addition, many of these series have included patients with primary tumor-related symptoms or complications at initial presentation[33,44,71].

With recent advances in systemic chemotherapy, the risks and benefits of immediate or deferred surgical strategy have changed. In contrast to the response rates of approximately 15% to 5-fluorouracil, combinations with modern chemotherapy regimens, such as infusional 5-fluorouracil/leucovorin with oxaliplatin or irinotecan, have yielded response rates of 50% and disease control rates of 85% in prospective clinical trials[72,73]. Furthermore, the addition of the targeted agents bevacizumab or cetuximab to the above combinations has provided clinically significant improvement in response rates[5,28,29,74]. In the setting of these effective chemotherapy regimens, the risk of primary tumor-related complications and the need of subsequent urgent intervention are low, less than 15% in most series (Table 4).

In series in which patients were mainly treated with effective chemotherapy (oxaliplatin, irinotecan, targeted agents) and had asymptomatic or uncomplicated primary tumor at presentation, the risk of complications was inferior to 10%, which can be explained by the significant tumor response to chemotherapy[21,75,76]. In addition, the risk of emergency colectomy for complications varies from 2% to 29%, with a rate of less than 7% in the two

Table 4 Complications related to in situ tumor in patients with unresectable stage IV colorectal cancer treated with chemotherapy as initial management *n* (%)

Ref.	No. of patients	Primary tumor-related complications (%)	Type of complication during chemotherapy			Surgery required for complication (%)
			Obstruction	Bleeding	Perforation	
Scoggins *et al*[82]	23	9	2 (9)	0	0	9
Sarela *et al*[71]	24	29	4 (17)	0	0	21
Ruo *et al*[44]	103	29	30 (29)	0	0	29
Tebbut *et al*[34]	82	23	11 (13)	3 (4%)	5 (6)	10
Michel *et al*[90]	23	22	5 (22)	0	0	22
Benoist *et al*[79]	27	15	4 (15)	0	0	15
Muratore *et al*[75]	35	8.5	2 (6)	1 (3%)	0	3
Galizia *et al*[38]	23	30	4 (17)	1 (4%)	2 (9)	17
Evans *et al*[70]	52	23	3 (6)	9 (17%)	0	2
Poultsides *et al*[76]	233	11	18 (8)	0	5 (2)	7
Karoui *et al*[33]	123	19	21 (17)	0	2 (2)	12
McCahill *et al*[77]	86	16	10 (12)	0	1 (1)	12

most recent series. In a series reporting 233 consecutive patients treated with primary chemotherapy, 26 (11%) patients developed a complication related to the primary tumor: colonic obstruction in 18 cases (9 effectively treated with a colonic stent), perforation in 5 cases, and pelvic pain in 3 patients with rectal cancer[76]. Among the 26 patients with a complication, only 16 (7%) required an intervention. In this series, no factor was correlated with the risk of primary tumor-related complication requiring an intervention under chemotherapy.

Lastly, in a phase Ⅱ trial, McCahill *et al*[77] recently reported a major morbidity rate of 16.3% (14 patients) in 86 patients with an intact primary tumor, receiving a chemotherapy by FOLFOX and bevacizumab. Primary tumor-related complications occurred in the first 12 mo following inclusion in 83.3% of cases. It consisted in 10 surgical interventions for primary tumor-related symptoms and two deaths attributed to complications of the intact primary. Among these 10 surgeries, indications were colonic obstruction in eight, perforation in one and abdominal pain in one. Six interventions were performed in emergency, three implicated performing definitive stoma and one postoperative death occurred. Four more patients had primary-related-complications, including two cases of bowel obstruction, which were managed without surgery, accounting for minor morbidity. In balance, 27 (31.4%) patients suffered from chemotherapy-related events and eight patients underwent a surgical resection with curative intent[77].

Although the expected risk is low, primary tumor-related complications may require urgent colonic stenting, or surgery with stoma creation, and may delay or even preclude chemotherapy administration. These risks should be clearly explained to patients before choosing between first-intention PTR or chemotherapy; and close follow-up performed to minimize their eventual proper consequences.

IS CHEMOTHERAPY-RELATED TOXICITY INCREASED IN THE PRESENCE OF THE PRIMARY TUMOR?

No specific studies have explored whether the presence or absence of the primary tumor could influence chemotherapy tolerance and safety. In the EORTC phase Ⅲ study[78], comparing perioperative FOLFOX chemotherapy with surgery alone, in patients with initially resectable liver metastases (≤ 4 metastases), no increased toxicity was reported in patients (34%) who had the primary tumor in place at the time of randomization. In several retrospective studies, no difference in chemotherapy-related toxicity was reported, regardless of whether the PT was in place or not[6,39,79].

Bevacizumab has been associated with a 1%-2% gastrointestinal perforation in prospective clinical trials[17,80]. Most bevacizumab-related perforations were observed in the first 3 mo of treatment, especially within the first month. It may occur throughout the entire gastrointestinal tract, including the site of the primary tumor. In the study reported by Poultsides *et al*[76] 48% of the patients received bevacizumab. Only two of the five perforations observed (all at the site of the primary tumor) occurred during bevacizumab therapy and one patient experienced perforation 6 mo after the last administration of bevacizumab, whereas two had never received it. Although the small number of patients who developed this complication may have precluded definitive conclusions, bevacizumab have not appeared to significantly increase the rate of perforation. Our group has reported similar results in a retrospective multicentric study[33]. In a recent study, among 86 patients receiving FOLFOX + bevacizumab without PTR, 23 (27%) had serious adverse events, including 4 (5%) chemotherapy-related deaths and 6 life-threatening toxicities[77]. Although not reported as serious adverse events but as primary tumor-related major morbidities, two patients had a bowel perforation, which was likely to be facilitated by bevacizumab.

For patients with *KRAS* wild-type tumor, anti-EGFR antibodies are also a possibility, although no study has yet examined the effect of these antibodies in metastatic CRC patients with the primary tumor in place[5]. Accordingly, in the particular case of colon cancer with unresectable SLM and a primary tumor in place, the literature does not currently justify a strategy different from that for CRC in general[81].

Table 5 Postoperative outcome after primary tumor resection in patients with unresectable stage IV colorectal cancer

Ref.	Study period	No. of patients	Mortality (%)	Morbidity (%)
Scoggins *et al*[82]	1985-1997	66	5	30
Rosen *et al*[43]	1984-1998	120	6	22.5
Tebbutt *et al*[34]	1990-1999	280	NM	13
Ruo *et al*[44]	1996-1999	127	2	21
Michel *et al*[90]	1996-1999	31	0	NM
Benoist *et al*[79]	1997-2002	32	0	19
Stelzner *et al*[45]	1995-2001	128	11.7	-
Galizia *et al*[38]	1995-2005	42	0	21
Evans *et al*[70]	1999-2006	45	16	NM
Bajwa *et al*[39]	1999-2005	32	3	22
Kleespies *et al*[46]	1996-2002	233	4.7	46
Mik *et al*[40]	1996-2000	52	7.7	40
Costi *et al*[48]	1994-2003	71	8.5	24
Stillwell *et al*[31]	1984-2004	379	9.2	48.3

NM: Not mentioned.

Overall, no data suggest that the presence of the primary tumor increases the toxicity of chemotherapy. Chemotherapy modalities, combined or not with targeted agents, should be the same as in the metachronous setting.

WHAT IS THE RISK OF COMPLICATIONS AFTER PALLIATIVE PRIMARY TUMOR RESECTION IN THE METASTATIC SETTING?

Several studies suggested that PTR was associated with high postoperative morbidity and mortality rates in the presence of metastases[12,45,82] (Table 5). One study reported that 15 of 128 patients (11.7%) patients died within 30 d postoperatively[45]. However, in this study many patients were symptomatic and underwent emergency surgery. The same series found a 27.8% mortality rate in patients who had emergency surgery *vs* only 7.3% mortality rate with elective procedure ($P = 0.002$)[45]. The high postoperative mortality rate of 5% reported by Scoggins *et al*[82] included patients who were symptomatic at the time of resection and the patient who died after surgery were noted to have severe carcinomatosis.

These mortality rates were higher that noted in the recently published meta-analysis where collectively, perioperative mortality was 1.7% (95%CI: 0.7-3.9)[8]. This lower mortality rate can be accounted for the preeminent number of patients that were asymptomatic and managed electively. In this meta-analysis, postoperative morbidity occurred in 23% (95%CI: 18.5-21.8) of patients. The most frequent complication was wound infection and could be mostly managed conservatively; however, in some instances, major complication arose whereby patients required additional surgery as management. Anastomotic leakage, occurring in 1.7% of patients, is more commonly a significant complication of rectal cancer resection. It often leads to sepsis, significantly prolongs hospital stay and delay or even precludes chemotherapy administration[8].

In a recent large monocentric series, this same group analyzed the postoperative outcomes in 379 CRC patients with unresectable synchronous metastases undergoing PTR[31]. In the postoperative period, mortality and morbidity rates were 9.2% and 48.3%, respectively. Postoperative surgical and medical complication rates were 35.6% and 25.3%, respectively. Among these patients, 33 required one or more reinterventions in the same admission to manage these complications. The most common surgical complications included wound infections and the most common medical complications comprised respiratory events followed by cardiac events. However, 45% of patients were aged of more than 70 years in this series, 60% had a locally advanced primary tumor and nearly 30% had rectal cancer[31].

These results need to be interpreted with caution as these studies suffered from several limitations. Firstly, morbidity rates were not always separated between minor and severe complications. Secondly, inclusion periods were very long and progresses in surgery and postoperative care have not been taken into account. In a recent series of 313 patients treated for unresectable synchronous stage IV CRC over different time periods, Platell *et al*[83] reported that the 30-d postoperative mortality (12.6% *vs* 2.7%, $P = 0.036$) and the duration of hospital stay (13 d *vs* 9 d, $P = 0.026$) have decreased significantly from 1996-2002 to 2003-2009 periods, despite increased numbers (28% *vs* 46.4%, $P = 0.001$) of patients with severe comorbidity (*i.e.*, ASA score 3 or 4). Another limitation resides in the heterogeneity of populations, as studied patients included those with symptomatic or locally advanced primary tumor, patients with rectal primary, patients with advanced age and severe comorbities, those with extensive and extra-hepatic metastatic spread or patients with poor general condition[8,31]. Fourthly, in all but two studies[31,46], there was no mention of the use of laparoscopy in patients electively undergoing PTR, which has been convinced to decrease postoperative morbidity compared to laparotomy. Indeed, in several phase Ⅲ trials, overall surgical morbidity following elective colectomy for cancer was 0.7%-3% and 20%-28%, in patients operated with laparoscopy and laparotomy, respectively[84]. Finally, one should note that in all series reporting the postoperative outcome after PTR in stage Ⅳ CRC patients, there was no mention of the use of perioperative immunonutrition which has also been demonstrated to improve postoperative outcomes in patients operated for various types of digestive cancers[85].

Few studies have performed a multivariate logistic regression analysis to determine independent factors associated with postoperative mortality and morbidity in patients with stage IV CRC. In the series reported by Stelzner *et al*[45] postoperative mortality (11.7%) was not associated with PTR but was significantly related to ASA score IV (ASA score III, 7% *vs* ASA score IV, 26.4%, $P = 0.002$), higher age (≤ 75 years, 7.6% *vs* > 75 years, 20%, $P = 0.015$) and emergency operations (27.8%, *vs* elec-

tive, 7.3%, $P = 0.002$). In the largest series of 379 resected patients with an unresectable stage Ⅳ CRC, Stillwell *et al*[31] found that at multivariate analysis, 30-d postoperative mortality was independently associated with medical complications ($P < 0.001$), emergency interventions ($P = 0.001$) and age (≥ 70 years, $P = 0.007$). Conversely, patients with liver-only metastases were less likely to die in the postoperative period than those with advanced local disease and/or extra-hepatic disease ($P = 0.004$). In this large series, emergency interventions were also linked to morbidity, a fact that is well established in literature[45,68-70]. In another series, independent determinants of an increased postoperative morbidity (total rate of 46%) were primary rectal cancer, hepatic tumor involvement > 50%, and comorbidity > 1 organ[46].

To summarize, after palliative PTR in metastatic patients, most studies suggested that baseline characteristics (age, WHO-PS, comorbidity, ASA score), advanced local and metastatic disease and rectal primary tumor to be related to postoperative morbidity and mortality. Taken together, these findings suggest that one issue for a phase Ⅲ study would be to assume that the acceptable risks of postoperative mortality and severe morbidity rates would be less than 10% and 30%, respectively. These rates could be even lower with the use of laparoscopic approach, which is known to improve short-term outcomes, including postoperative morbidity, compared to open surgery[23-25,86]. Besides, perioperative nutrition should be systematically recommended. Finally, these anticipated morbidity and mortality rates are those expected in a population of selected patients, constituted after the exclusion of patients which would not be likely to benefit from PTR (patients in poor general condition, with severe comorbidities, rectal cancer, extra-hepatic metastatic disease, complicated primary tumor).

SPECIFIC ISSUES OF RECTAL CANCER

By its particular location in the pelvis, rectal cancer differs from colon cancer on several points: first, unresected rectal tumors can lead to disabling symptoms (pelvic pain, rectal syndrome) and local related complications such as urinary obstruction, perforation with pelvic abscess or recto vaginal fistula that can be disastrous and difficult to manage; secondly, for locally advanced mid and/or low rectal tumors (*i.e.*, staged cT3, T4 and/or cN-positive disease) neoadjuvant treatment (short-course radiotherapy (RT) or long-course chemoradiotherapy) has been demonstrated to decrease the risk of local recurrence with no effect on survival; finally rectal resection with total mesorectal excision is a demanding surgery with high postoperative complications rates (which may delay or even preclude chemotherapy administration), risk of long-term functional disorders (digestive, sexual, urinary) that can negatively impact on quality of life and lead to permanent stoma in up to 20% of operated patients[87].

In patients with rectal cancer and synchronous unresectable metastases, up-front chemotherapy administration before considering the need to resect the primary tumor may represent an attractive therapeutic option for the following reasons: surgery (with or without neoadjuvant treatment) is avoided in patients with rapidly progressive metastatic disease which should be regarded as a biological marker for poor prognosis and an indication for administering second-line treatment. In a retrospective study of 22 patients with rectal cancer and unresectable synchronous metastases, Stelzner *et al*[88] reported that, in patients without progression under first-line chemotherapy, median OS was significantly increased in patients who underwent PTR compared to those with the primary tumor left in place (27.2 mo *vs* 12.4 mo, $P = 0.017$). In addition, systemic chemotherapy has also an effect on primary tumor in rectal carcinoma. In a phase 2 trial evaluating neoadjuvant capecitabine and oxaliplatin before chemoradiotherapy and total mesorectal excision in 105 patients with locally advanced rectal cancer, Chua *et al*[89] emphasized that morphological reevaluation after neoadjuvant chemotherapy showed an objective response in 78 patients (74%). Based on these results, patients could receive short-course RT or even no RT at all before rectal surgery in case of partial or complete radiological response after neoadjuvant chemotherapy. In conclusion, for patients with rectal cancer and unresectable SLM, it seems relevant that chemotherapy should be the first treatment and surgery should only be proposed when there is no progression during preoperative chemotherapy. Patients with a poor prognosis due to progressive metastatic disease are thereby spared the risks of major rectal surgery with a long hospital stay and unnecessary surgical complications.

DISCUSSION: WHAT DESIGN FOR A STUDY ATTEMPTING TO ANSWER THIS ISSUE?

Whether PTR should be performed prior chemotherapy administration in unresectable stage Ⅳ CRC patients remains unknown. When the primary tumor is not resected and uncomplicated (asymptomatic) and the patient has started with palliative chemotherapy, the rate of unplanned or emergency surgery is relatively low and therefore does not warrant surgery of the primary in future patients. This relative low rate of primary tumor-related complications under chemotherapy may be partly explained by the effectiveness of chemotherapy regimens and targeted agents. With regard to survival, most retrospective studies favor PTR, but results are likely to be influenced by selection biases. These studies suggested that liver burden > 50%-75%, extra-hepatic metastatic disease (peritoneal carcinomatosis, lung metastases), advanced age and poor WHO-PS were poor prognostic factors in CRC patients with unresectable SLM even for those who undergo PTR. These factors, in addition to rectal primary location, have also been reported to be associated with high postoperative mortality and morbidity following PTR. In summary, data from the literature highlight that patient selection taking into account all the above men-

tioned factors is a critical issue for a future randomized trial aiming to determine whether OS is improved by PTR in patients with CRC and unresectable liver metastastic.

Definition of metastases unresectability is also a critical issue. Among patients with CRC liver metastases, no consensual precise definition of resectability or unresectability has been reached to date[3]. The resectability of liver metastases may differ from one hospital to another, depending on the available equipment and the level of surgical expertise. The definition also depends, understandably, on patient-specific data, such as general health, comorbidities, nutritional status, and more specifically, the presence of a possible underlying liver disease. For these reasons and to provide a rigorous framework, a relevant definition of liver metastases unresectability would be the inability to achieve a macroscopically complete resection (with clear margins) of all metastases, in one- or two-stage, without compromising postoperative liver function because of the insufficiency of either the remaining liver volume or biliary and venous vascularization and drainage. Unresectability of liver metastases would have to be assessed on a helical or multi-slice abdominal CT-scan with contrast enhancement, or liver MRI if CT is impossible (kidney failure, allergy to iodine) or insufficient to characterize lesions[81]. Radiological criteria for liver metastases unresectability would gather involvement of all hepatic veins, or both portal branches, or one portal branch and the controlateral hepatic vein(s), and a predictable post-hepatectomy liver volume < 25%-30%.

Then, all eligible patients would be randomized to undergo either PTR followed by chemotherapy ± targeted agent or chemotherapy ± targeted agent without PTR. Randomization would be stratified according to the study center and the metastatic liver involvement (≤ 50% *vs* > 50%) as determined by the pretreatment CT-scan or liver MRI staging.

The primary endpoint would be the difference in OS between the two treatment arms. Secondary endpoints would be quality of life, rate of primary tumor-related complications in the arm with chemotherapy alone and postoperative morbidity in the PTR arm. Besides, the tolerability of chemotherapy, objective tumor response, PFS, time to metastatic progression and the rate of secondary curative resection (R0) of both the primary and metastases should be assessed in both treatment arms.

No randomized study has been performed yet. The entire international community wishes to answer this question. One should emphasized that since 2010 until today, 14 papers on the present subject have been published including 9 individual series, 5 reviews or meta-analyses, 1 editorial and 1 guidelines from the French authorities. In all these publications, the need to perform a randomized trial evaluating the impact of PTR on survival in patients with CRC and unresectable metastases is underlined.

CONCLUSION

The present review assessed whether OS and quality of life are improved in patients with asymptomatic unresectable metastatic CRC treated with surgery followed by chemotherapy *vs* chemotherapy alone with the primary in place. Reported data from the literature support the view that PTR should be discussed and validated by a phase III trial in selected patients: asymptomatic primary tumor, age ≤ 70 years, WHO-PS < 2, no extra-hepatic metastatic disease, liver burden of less than 50%. In these patients, PTR, when performed laparoscopically and after preoperative immuno-nutrition, may lead to an increased OS. In all other cases, reported postoperative mortality and morbidity rates related to PTR are high and up-front chemotherapy with the primary tumor left in place may represent the more reasonable option.

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MINIREVIEWS

Monoclonal antibodies that target the immunogenic proteins expressed in colorectal cancer

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Abstract

In an attempt to improve upon the end results obtained in treating colorectal cancer it was apparent that the earlier the diagnosis that could be obtained, the better the chance for obtaining desired results. In the case of more advanced tumors typified by later stage colorectal cancer, surgical debulking is an important part of the treatment strategy. Here the use of additional therapeutic modalities including chemotherapy and present day immunotherapy has failed to accomplish the desired improvements that have been sought after. Adjuvant therapy, has offered little to the overall survival. The concept of early detection is now recognized as the initial step in reaching proper end results and can readily be demonstrated from colorectal cancer studies. Here survival has been found to be a reflection of the stage at which the tumor is first identified and treated. When specific monoclonals targeting colorectal cancer are employed diagnostically, we have been able to demonstrate detection of colorectal cancer at its inception as a premalignant lesion, such that genotypic features can be identified before the phenotypic appearance of cancer can be noted.

Key words: Colorectal cancer; Monoclonal antibodies; Immunohistochemistry; Antibody dependent cell cytotoxicity; Tumor associated antigens

Core tip: The ideal monoclonal antibody to be employed in cancer management is one targeting an immunogenic protein expressed in a specific cancer system. Those presently employed in cancer management, target a growth factor or carbohydrate antigen seen in both cancer and normal tissue. Their value as such is limited. The monoclonals described herein are directed against colon cancer tumor associated antigen and have value in both diagnostic and therapeutic uses for controlling this disease.

Arlen M, Arlen P, Coppa G, Crawford J, Wang X, Saric O, Dubeykovskiy A, Molmenti E. Monoclonal antibodies that target the immunogenic proteins expressed in colorectal cancer. *World J Gastrointest Oncol* 2014; 6(6): 170-176 Available from: URL: http://www.wjgnet.com/1948-5204/full/v6/i6/170.htm DOI: http://dx.doi.org/10.4251/wjgo.v6.i6.170

INTRODUCTION

For most malignancies such as colorectal cancer, the earlier the diagnosis the better the chance for offering the patient the opportunity to be cured[1-5]. The addition of additional methods to help improve survival, especially in the post operative period, have offered little to achieve a better response[6-9]. In order to define methods for earlier intervention, we began to look at behavioral patterns seen in various stages of colorectal cancer, attempting to define those patterns related to tumor antigen expression. We were able as such, to identify and characterize a unique group of immunogenic proteins that appeared

to be expressed in all colorectal carcinomas that were examined. These tumor (associated) antigens were found to be present in all stages of colorectal tumor development, from inception to metastasis. Following the separation of these proteins from pooled tumor cell membranes, monoclonal antibodies targeting these proteins were developed. Hybridomas were produced by injection of BALBc mice with the antigens/proteins so obtained.

By employing those monoclonal antibodies derived against the tumor proteins, it appeared that the antigen, noted to be expressed in the earliest stages of tumor development, continued to be present throughout later stages of progression of tumor growth. As a result, we were able to define the appearance of genetic alterations occurring in normal appearing cells that first characterized the transformation process. This initial pattern of cellular transformation was typified by the expression of immunogenic tumor proteins in the earliest stages of genotypic transformation when phenotypic features still appeared normal by standard HE. As with the invasive cell which sheds its antigen into the serum, the premalignant cell similarly sheds antigen into the stool which can easily be identified by a stool enzyme-linked immunosorbent assay (ELISA). Tissue biopsies studied by immunohistochemistry to define cells expressing tumor antigen and examination of stool for the presence of tumor antigen can now offer the asymptomatic patient the opportunity for proper screening. As a result one can now offer a practical process for early detection of a developing malignancy when optimum results can almost always be anticipated.

We now believe that it is possible to define the presence or absence of colon cancer during the screening process of the asymptomatic patient. If validated by our studies, the need to employ colonoscopy would be markedly reduced and relegated to those patients where there is a high likely hood for defining an early malignancy or when biopsy is required for confirmation of as well as staging of the disease process.

As noted above, the early premalignant cells undergoing transformation, as well as polypoid tumors and larger malignancies do shed tumor antigen into the stool where they can be detected by stool ELISA using our colon tumor monoclonals. This procedure can be used as a confirmatory measure to determine whether colonoscopy is or is not indicated as a follow up in post op patients in order to detect early developing lesions as well as possible anastomotic recurrences.

These same antibodies, used for detecting colon specific tumor associated antigens, also have therapeutic efficacy. Should the clinical work up of a malignant lesion demonstrate spread of tumor, the monoclonals that were employed for diagnosis of the tumor marker, can now be delivered intravenously to target those cells producing tumor antigen and destroy them through the process of antibody dependent cell cytotoxicity (ADCC).

DISCUSSION

At the present time, most of the tumor markers employed commercially for tumor detection and diagnosis are non specific. Those clinically available, best serve to monitor the response to the therapy being employed rather than to detect and diagnose the presence of a lesion. Those markers that appear in the serum are mostly derived from carbohydrate antigens that are shed into the serum. They are not only expressed by the tumor, but also by adjacent normal tissue that may have been effected by an ongoing inflammatory process[10-12].

In order to detect the presence of colon tumors at the ideal time, it is important to be able to define a specific marker or family of markers on the tumor when clinical symptoms were minimal if not totally absent. Such markers have been shown to best be represented by one or several immunogenic proteins or glycoproteins expressed on the cell surface membrane and found to shed into the serum as well as surrounding tissue. Those immunogenic proteins that characterize colon cancer have been isolated and characterized by our group at Precision Biologics. Pooled allogeneic specimens of colon cancer were used to retrieve tumor membrane proteins, separate them by molecular weight and then skin test the patient to define that specific group of proteins producing delayed cutaneous sensitivity. Further separation by isoelectrophoresis yielded three distinct glycoproteins that proved to represent oncofetal proteins first expressed in the fetus and later in a mutated form, representing specific colon cancer proteins that help induce a mild immune response. The failure to achieve a full immune response proved to be due to minimal expression of antigen in the tumor that was necessary to induce a proper immune response.

Using monoclonal antibodies developed against these immunogens, a serum ELISA was also developed that is capable of identifying shed markers with a high degree of sensitivity and specificity[13]. The monoclonal antibodies that specifically target these tumor proteins, have demonstrated that these proteins serve both as diagnostic markers and a therapeutic targets[14].

It is well known that of the many methods being developed to control the more aggressive colon lesions, not only does one rely on newer chemotherapeutic agents, but additionally through enhancement of the immune system. This can be accomplished by combining chemotherapy with a monoclonal antibody such as the one directed against the epidermal growth factor 1[15]. The process of adding an immunotherapeutic agent to standard chemotherapeutic drugs does rely on the nature of the antigen expressed by the tumor. This of course can be accomplished by immunohistochemical analysis of the tumor. The same effective monoclonal antibody that detected the presence of the tumor antigen/marker in the biopsy specimen can then be used intravenously along with chemotherapy, to attack the marker as a therapeutic target. In such combinations, the chemotherapeutic agent may serve to minimize the presence of any shed blocking material from the tumor to secondarily enhancing the immune response. Such enhancement in immune reactivity frequently helps the host defense mechanisms to control

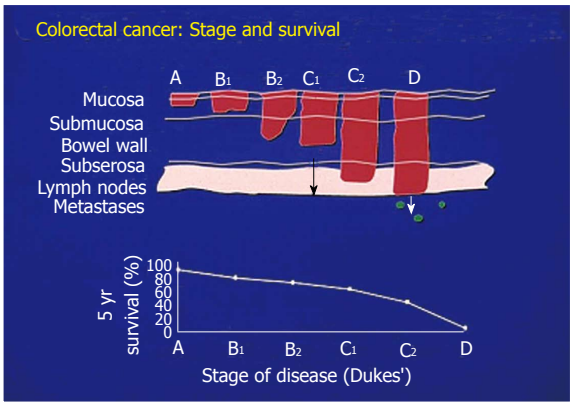

Figure 1 Correlating the extent of local tumor progression with survival in colorectal cancer.

disease progression[16-19].

When a primary colon tumor is confined to the mucosa of the bowel, cure is just about guaranteed by surgical removal. However, when the tumor is found to penetrate into the muscular layers of the bowel, or invades the serosal surface with regional nodes possibly being involved, the opportunity for cure diminishes (Figure 1). Here additional modalities of therapy are essential if improvement in survival is to be accomplished.

The size of a tumor mass becomes part of the overall picture of how the lesion is viewed regarding its management. A greater host immune response is required in the more advanced cases as typified by bulky disease. This almost always necessitates surgical debulking to eliminate the larger number of tumor cells that are required to be brought under control. The presence of bulky tumor is in addition, frequently associated with a source of inhibitory surface molecules. When shed from the tumor cell membrane into the serum, these molecules function to inhibit those immunosurveillance mechanisms needed for helping to eliminate existing tumor cells that may have remained in the region of surgical resection or among those cells having entered the circulation[20]. As a consequence, a greater host immune response is required in the more advanced cases which is usually typified by bulky disease. There is little disagreement as such, that the ability to achieve an improved cure rate depends on early diagnosis and when possible, complete removal of the existing tumor.

The concept for achieving the early diagnosis of a malignant lesion was espoused by Lee Hartwell of the Fred Hutchinson Cancer Center, who evaluated procedures for achieving such early diagnosis as the more effective way of curing cancer. He looked at later stages of disease in solid tumor malignancies, where chemotherapy was employed to help improve survival. In such situations he found that this approach rarely resulted in cure, especially when the primary lesion had undergone the process of metastasis[21].

Hartwell stressed the need for finding a tumor protein expressed early in the onset of disease, functioning in a manner that the Pap smear had accomplished for cervix cancer. When such a tumor protein, functioning as a marker, could be detected by a monoclonal antibody, the clinical course of the disease would be altered in favor of an almost guaranteed cure. Larry Norton of the Sloan Kettering Cancer Center emphasized that if the tumor markers that Hartwell was hoping to find were immunogenic, then the monoclonal antibody that could determine the presence of the malignant lesion would be the same monoclonal that when delivered intravenously, would hunt, seek and destroy any cell in the metastatic setting that presented with such a marker. Essentially the presence of immunogenic tumor associated antigens (TAA's) on the cell surface membrane serve to illustrate the tumor in the form of a coin displaying two sides. On the reverse side of the coin, the proper monoclonal can detect the tumor antigen as a diagnostic marker. The antigen on the opposite (head) side of the coin would now act as a therapeutic target for tumor destruction by utilizing the same monoclonal antibody delivered intravenously (Figure 2).

Such tumor immunogenic proteins (TAA's) were isolated from a number of different malignancies including colon cancer and later characterized at Precision Biologics. The monoclonals that were derived from colon cancer antigen and later used to immunize BALBc mice for hybridoma production, are presently being tested clinically for both diagnostic and therapeutic efficacy. They have been found to be capable of detecting the earliest lesion in a manner illustrated by Figure 2. These colon tumor specific monoclonals are capable of functioning to diagnose the presence of the colon malignancy by both immuno-histochemistry of the resected specimen as well as serum ELISA. Should the tumor have invaded the blood stream, the metastatic lesions resulting from such invasion can now be effectively targeted. Extrapolating from animal studies with colon cancer transplants, metastatic foci from of these tumors can now be approached thru intravenous infusion of the monoclonal antibody with doses of the IgG1 delivered Ⅳ at 4-5 mg/kg. Phase Ⅱ B studies are now in progress with these antibodies.

When Ariel Hollinshead (1985)[22] employed pooled allogeneic tumor membrane antigen for treating a variety of malignant lesions, it became apparent that when the antigen was delivered at threshold levels and specifically for the malignancy expressing suboptimal levels of innate antigen, that the immune system could be shifted from one of performing immune-surveillance to that of providing a therapeutic mechanism for attacking and destroying the tumor, resulting in improvement in survival[23,24].

Clinical studies employing pooled allogeneic tumor antigen in the form of a vaccine, defined by its ability to turn on both cell and humoral immunity, resulted in improved survival over those where patients underwent surgery alone. In order to achieve an optimum response, the antigen had to be delivered at doses of between 750 and 1000 μg in 3 divided doses, given along with an oil based adjuvant. This allowed the now homogenized antigen to remain at the site of delivery for an extended period of

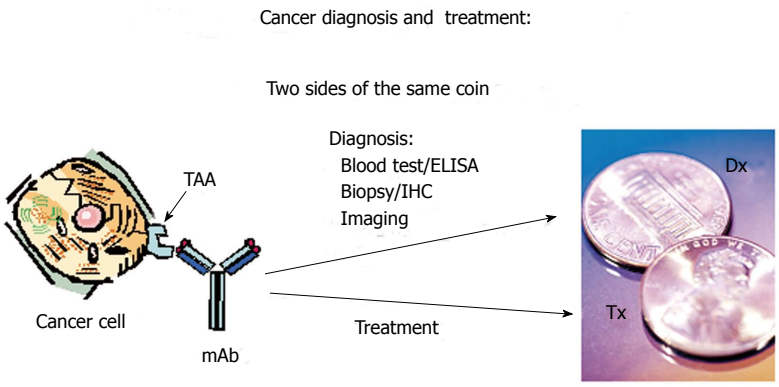

Figure 2 Depicts the ideal monoclonal antibody that can define the presence of a tumor associated antigen for diagnosis by Immunohistochemistry and then when delivered Ⅳ, can hunt and destroy the tumor which contains the diagnostic marker. IHC: Immunohistochemistry; TAA: Tumor associated antigen; ELISA: Enzyme-linked immunosorbent assay.

time.

To define the nature of the tumor protein or proteins capable of inducing enhancement in tumor recognition, monoclonals were developed in BALBc mice. Three of the antibodies obtained from the fusion and subsequent hybridoma development showed specificity for colon cancer. There was minimal if any evidence of cross reactivity of these antibodies to the surrounding normal colonic tissue. When employed for therapy, first chimeric and then the humanized or human version of the antibodies were produced.

In reviewing the nature of the clinical response obtained following the initial trials employing pooled colon cancer antigen, all patients immunized had a strong delayed cutaneous hypersensitivity response as previously noted. This was response was associated with enhancement in cellular immunity as well a strong humoral response in most patients, with resulting high serum titers of an IgG1 targeting the antigen expressed on the tumor cells[25]. Those among the 10%-20% of patients showing signs of recurrent disease after immunization, were found to be unable to mount the needed humoral response needed to control the tumor. The cell lmediated immunity almost appeared to function in a bystander manner. The monoclonals described above that were developed from the original Hollinshead tumor antigen were then specifically produced GMP for initiation of food and drug administration (FDA) clinical trails. The IgG1 format developed for the trials was found to function in the same manner as those antibodies found in the host circulation in response to administration of the tumor vaccine.

A detailed analysis of the monoclonals so produced against the colon antigen revealed each to be capable of inducing a strong ADCC response. Similarly, these mAbs showed effectiveness in a serum ELISA with a high degree of sensitivity and specificity. Using Immunohistochemistry (IHC) to define expression of antigen in the tissue under examination, cells that have undergone the initial genotypic changes can now be clearly defined even though the phenotypic features of cancer are not yet available for recognition by the pathologist. Studies to date have suggested that the colonocytes adjacent to a malignant lesion, have for the most part undergone genotypic transformation (Figure 3). It appears that this process of malignant transformation occurs several months before phenotypic features of cancer can be detected[26]. Obviously during resection of a primary colon lesion by colectomy, it is essential for the pathologist to guarantee that transformed colonocytes not be left behind in the margins of resection that are to be re-anastomosed. This appears to be best achieved by employing IHC with the monoclonal antibodies targeting colon tumor antigen. Along with the standard HE protocol. We plan to have antibody kits available in the OR so that frozen sections taken from margins of bowel following colectomy can be obtained for IHC.

Tumor antigen structure was analyzed, defined and characterized following immunoprecipitation of the pooled allogeneic colon cancer membrane material that had been used as a vaccine. Mass spectroscopy indicated that there were three separate antigens, seen alone and in combination in various colon cancers, each representing an oncofetal protein needed in the development of the human GI tract. These proteins were usually turned off as the fetus matured by re: methylation of the gene. In the adult, the onset of malignant transformation of the cell occurs *via* an oncogenic mutation. This appears to result in a modification of the protein structure through a mutation in the synthetic pathway or possibly thru a post translational modification of the oncofetal protein. The resulting tumor protein was found then to be immunogenic and serves to characterize the tumor system in which it is expressed. The immunogenic proteins that we identified were shown to be related to MUC5ac, A33, and CEAcam 5,6. While our monoclonals clearly define these proteins on Immunohistochemistry, commercial monoclonals used to define the known non modified antigens (oncofetal proteins) failed to recognize expression of the modified antigen in the malignant system.

All of the monoclonals that we have developed fit into a unique class of IgG's that are both diagnostic as well as therapeutic in solid tumor malignancies. Mutated MUC5c antigen is defined by monoclonal Neo-101 and its newer version Neo 102, CEAcam5,6 by monoclonal 16C3/Neo 201 and altered A33 by monoclonal 31.1. To date no other anti-tumor IgG monoclonals have been found capable of performing in a similar fashion. The epidermal antibodies targeting epidermal growth factor I and II all have corresponding targets in normal tissue.

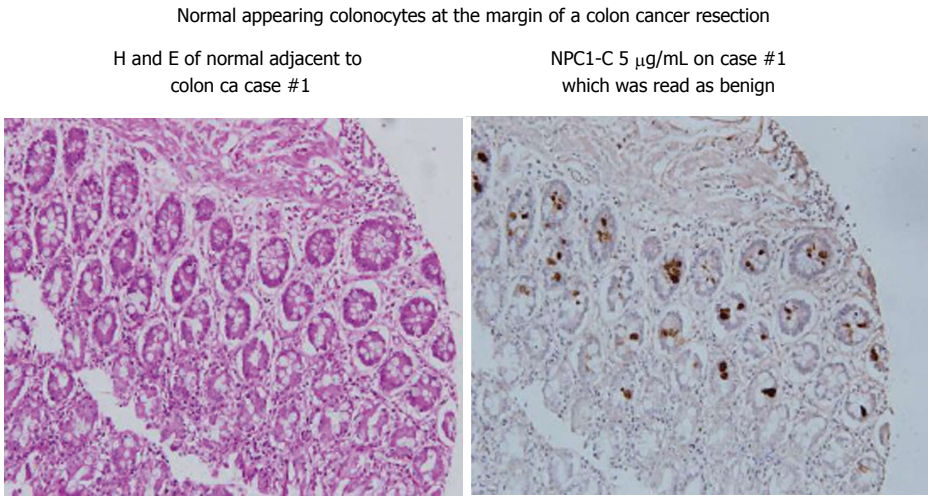

Figure 3 Reveals expression of tumor antigen in those colonocytes adjacent to the malignant lesion where the colonocytes appear normal by H and E.

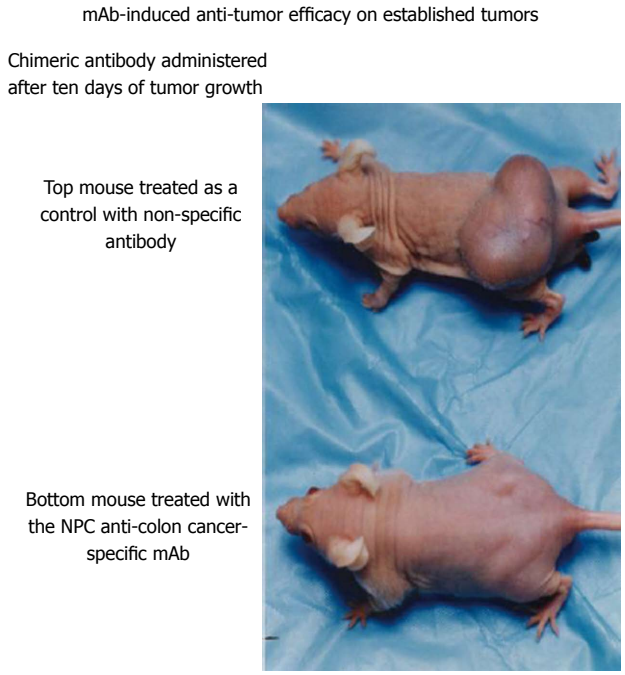

Figure 4 Animal models (nude mice) growing human malignancy to compare untreated and treated animals. The upper model received a control mAb while the lower animal model having had a much smaller tumor mass at 10 d received mAb NPC-1.

Knowing that the targeted antigen in colorectal cancer can result in tumor destruction, animal studies prior to initiation of clinical trials using therapeutic monoclonals, were devised to demonstrate *in vivo* tumor destruction Figure 4.

The ADCC response for most of the Precision monoclonals, range from 50%-70% tumor destruction in a 6-8 h. period of time, at an effector to tumor (E:T) ratio of 80-100:1 to over 90% with monoclonal 31.1. When these antibodies are delivered intraperitoneally in the animal model following establishment of tumor growth 10 d after subcutaneous administration of 10-20 million tumor cells in the thigh of nude mice, more than 50% of the animals were found to have a marked reduction in size the tumor mass. This can be seen at 10-15 d after immunization. The dosage of intraperitoneal IgG delivered along with human effector cells to assure an optimum ADCC response, was found to require approximately 400 μg in the animal model or an equivalent of approximately 400 mg in a 70 kg patient, this represents about 4-5 mg/kg of monoclonal antibody delivered at about 1 mg/min.

Considering the lack of toxicity following Ⅳ administration of our monoclonals in phase Ⅰ FDA therapeutic trial, we began phase Ⅱ studies. One of the problems encountered in the original GMP antibody preparation for FDA was that NEO-101 mAb was expressed at low levels and therefore not suitable for commercial production. Using a newer expression system, we are now able to produce the new monoclonal at a significantly higher level. Of interest was that while the sequence of the newly produced antibody, NEO-102 was virtually unchanged, we did see an approximate a definite improvement in ADCC as well as improvement in the quality of staining where background staining was virtually eliminated. This new version of the mAb, NEO-102 is being utilized in phase Ⅱ and is being tested in escalating doses. Phase Ⅱb has been designed to test the optimum dose of NEO-102 in combination with chemotherapy[27].

As mentioned above, the antibodies developed at Precision Biologics have their clinical efficacy in their capability of defining the tumor marker expressed in the tumor cell as a target for tumor detection as well as destruction. In tracing the pattern of expression of these markers, it became readily apparent that they were expressed not only in the later stages of tumor development where they could serve as an ideal therapeutic target, but at a time when genotypic changes were taking place in the normal but transforming cell, as noted above, and where the features of malignancy could not be readily recognized by the pathologist. We are now looking at the issue of Field Effect with regard to the genetic alterations occurring at the time of tumor induction. As such we are attempting to define the extent of premalignant

alterations surrounding the primary lesion[28].

In terms of colon cancer, the mechanism for tumor induction whether by virus or carcinogen, probably effects an area in the bowel resulting in a pattern of genotypically altered colonocytes expressing tumor antigen, the so called Field Effect as noted above. Within this Field, further mutations lead to the eventual appearance of the early polypoid changes that may suppress the genotypically altered surrounding colonocytes. This polypoid lesion then continues with further mutational changes leading to the eventual appearance of an infiltrating colonocytic lesion. Resection of the polypoid lesion, leaving the altered colonocytic field intact, could then result in further progression of cellular changes in the premalignant cells. Such a concept, if proven correct as per an ongoing study at North Shore University Hospital and Precision Biologics will assist the pathologist, at the time of bowel resection, to define the extent of the Field Effect by immuno histochemistry.

In our ongoing therapeutic trials, phase Ⅱb is in the process of initiation with the addition of chemotherapy to the therapeutic monoclonals being employed. It is generally agreed upon that Immunochemotherapy can be more effective than either chemotherapy or immunotherapy when employed alone. In general chemotherapy can diminish the immune inhibitory effect derived from the tumor and enhances the overall therapeutic response[29,30]. Finally we have prepared an alpha particle labeled NEO-102 monoclonal antibody to be introduced at a later date as part of the overall therapeutic approach to tumor control.

The availability of monoclonals targeting an immunogenic protein expressed in all phases of colon cancer development should be useful for both diagnosis and therapy and should have a major impact on how colon cancer is treated and the outcome that can be expected.

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SYSTEMATIC REVIEW

Current status of pharmacological treatment of colorectal cancer

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Abstract

AIM: To review the clinical trials for the development in drugs for chemotherapeutic treatment of colorectal cancer (CRC).

METHODS: A systematic review identified randomized controlled trials (RCTs) assessing drugs for the treatment of CRC or adenomatous polyps from www.clinicaltrials.gov. Various online medical databases were searched for relevant publications.

RESULTS: Combination treatment regimens of standard drugs with newer agents have been shown to improve overall survival, disease-free survival, time to progression and quality of life compared to that with standard drugs alone in patients with advanced colorectal cancer. The FOLFOXIRI regimen has been associated with a significantly higher response rate, progression-free survival and overall survival compared to the FOLFIRI regimen.

CONCLUSION: Oxaliplatin plus intravenous bolus fluorouracil and leucovorin has been shown to be superior for disease-free survival when compared to intravenous bolus fluorouracil and leucovorin. In addition, oxaliplatin regimens were more likely to result in successful surgical resections. First line treatment with cetuximab plus fluorouracil, leucovorin and irinotecan has been found to reduce the risk of metastatic progression in patients with epidermal growth factor receptor-positive colorectal cancer with unresectable metastases. The addition of bevacizumab has been shown to significantly increase overall and progression-free survival when given in combination with standard therapy.

Key words: Colorectal cancer; Metastasis; Chemotherapy; 5-fluorouracil; Leucovorin; Epidermal growth factor receptor inhibitor

Core tip: A systematic review was undertaken to identify randomized controlled trials (RCTs) assessing synthetic drugs for the treatment of colorectal cancer and/or adenomatous polyps from various medical databases, including clinicaltrials.gov, and a total of around 2300 RCTs were screened. After reviewing data from RCTs of synthetic drugs, alone or in combination with biological agents, for the treatment of colorectal cancer, it was concluded that combination regimens of standard chemotherapeutic drugs with new cytotoxic and targeted agents have led to an increase in overall and progression-free survival and have also contributed to increased rates of resectability and improved health-related quality of life in patients.

Akhtar R, Chandel S, Sarotra P, Medhi B. Current status of pharmacological treatment of colorectal cancer. *World J Gastrointest Oncol* 2014; 6(6): 177-183 Available from: URL: http://www.wjgnet.com/1948-5204/full/v6/i6/177.htm DOI: http://dx.doi.org/10.4251/wjgo.v6.i6.177

INTRODUCTION

Colorectal cancer (CRC) is a malignant neoplasm arising from the lining of the large intestine (colon and rectum). It is the third most common cancer in males and the second in females. Countries such as Australia, New Zealand, Canada, the United States and parts of Europe have the highest incidence rates, whereas China, India, parts of Africa and South America have the lowest risk of colorectal cancer in the world[1]. This geographical variation in incidence across the world can be attributed to differences in the consumption of red and processed meat, fiber and alcohol as well as body weight and physical activity[2-7]. However, the incidence of colorectal cancer is increasing in Japan and other Asian countries as there has been a shift towards westernized diets and lifestyles[2]. The survival rate for colorectal cancer varies with stage of disease at diagnosis and typically varies from 90% for cancers detected at the localized stage to 10% for distant metastatic cancer. The incidence of colorectal cancer has been known to increase with age. The likelihood of colorectal cancer diagnosis increases progressively from a younger age (< 40 years) and rises sharply after the age of 50 years[8,9]. Several factors such as poor quality diets[10], lack of physical activity, obesity[11], cigarette smoking[12] and heavy alcohol consumption[13] are associated with an increased risk of colorectal cancer. An individual with a history of adenomatous polyps or inflammatory bowel disease has an increased risk of developing colorectal cancer compared to an individual with no history of either[12,14].

Colorectal cancer includes malignant growths from the mucosa of the colon and rectum. Cancer cells may eventually spread to nearby lymph nodes and subsequently to more remote lymph nodes and other organs in the body like the liver and lungs, among others. The treatment, prognosis and survival rate largely depends on the stage of disease at diagnosis. Screening for colorectal cancer is particularly effective. Screening can prevent cancer from occurring as it can detect adenomatous polyps that can be successfully removed[15]. Treatment for colorectal cancer varies by tumor location and stage at diagnosis. Surgical removal of tumor and nearby lymph nodes is the most common treatment for early stage (stage I or Ⅱ) colorectal cancer. For patients with late-stage disease, chemotherapy alone or in combination with radiation therapy is often given before or after surgery.

MATERIALS AND METHODS

A systematic review was undertaken to identify randomized controlled trials (RCTs) assessing drugs for the treatment of colorectal cancer and/or adenomatous polyps from www.clinicaltrials.gov. Trials with unknown status were excluded. The following electronic databases were searched for RCTs of clinical effectiveness: MEDLINE, Medline In-Process and EMBASE. A separate literature search was undertaken to identify relevant articles from various online databases such as PubMed. The search was conducted using the following key words and phrases: colon cancer, colorectal cancer, clinical trials and drugs in colon/colorectal cancer.

RESULTS

The search identified 1663 RCTs of synthetic drugs, alone and/or in combination with biological agents, including on-going, completed and suspended/withdrawn/terminated studies in colorectal cancer.

Fluoropyrimidines

Fluoropyrimidines are anti-metabolite agents widely used in the treatment of various cancers. The principal mechanism of action of fluoropyrimidines has been considered to be the inhibition of thymidylate synthase. The response to 5-fluorouracil (5-FU) as a first line monotherapy is low, so it is given in combination with other cytotoxic agents, like oxaliplatin and irinotecan. 5-FU is commonly given either as a bolus injection with leucovorin (folinic acid) or a continuous infusion. While 5-FU bolus treatment favors RNA damage, continuous treatment with 5-FU favors DNA damage[16]. 5FU when given orally is associated with unpredictable levels in the plasma with extensive interpatient and intrapatient variability[17]. The primary cause of variability in plasma levels is the extensive first pass metabolism of the drug in the gut wall and liver. It was also thought to result from its erratic intestinal absorption due to a difference in concentration of dihydropyrimidine dehydrogenase or DPD (rate-limiting enzyme involved in 5-FU metabolism) in the mucosa. This problem can be overcome by administration of a fluorouracil that is not catabolized by DPD[18] and the coadministration of oral fluorouracil with an inhibitor of DPD[19]. Prodrugs of 5-FU are absorbed intact through the gastrointestinal mucosa and undergo enzymatic activation by one or more enzyme systems to release 5-FU intracellularly.

Multi-drug chemotherapy

The Gruppo Oncologico Nord Ovest (GONO) conducted a phase Ⅲ study involving 244 patients with previously untreated metastatic CRC, comparing fluorouracil, leucovorin, oxaliplatin and irinotecan (FOLFOXIRI) with infusional fluorouracil, leucovorin and irinotecan (FOLFIRI). The results of the study demonstrated that the FOLFOXIRI regimen was associated with a significantly higher response rate, progression-free survival and overall survival compared to the FOLFIRI regimen[20]. In a phase Ⅱ study of 44 patients with unresectable metastatic colorectal cancer, neoadjuvant chemotherapy with fluorouracil, leucovorin and oxaliplatin (FOLFOX4) was associated with a high response rate, thus allowing for successful resection of disease in a portion of patients[21].

Oxaliplatin is a diaminocyclohexane platinum compound that acts by impairing DNA replication and induces cellular apoptosis[22,23]. In the National Surgical Adjuvant Breast and Bowel Project C-07 trial involving 2409 patients, oxaliplatin plus intravenous bolus fluo-

rouracil and leucovorin was superior for disease-free survival (HR = 0.82; 95%CI: 0.72-0.93; P = 0.002) when compared to intravenous bolus fluorouracil and leucovorin. Treatment with oxaliplatin significantly improved overall survival in patients younger than 70 (HR = 0.80; 95%CI: 0.68-0.95; P = 0.013), while no positive effect was evident in older patients. In this study, treatment with oxaliplatin in patients > 60 years and females was associated with increased incidence of bowel wall injury[24]. In another trial involving 2246 patients who had undergone curative resection for stage Ⅱ or Ⅲ colon cancer, the rate of disease-free survival at three years was 78.2% (95%CI: 75.6-80.7) in the group given fluorouracil and leucovorin (FL) plus oxaliplatin and 72.9% (95%CI: 70.2-75.7) in the FL group[25]. In the National Cancer Institute-sponsored trial N9741 involving 1508 patients with locally advanced or metastatic colorectal cancer, oxaliplatin plus fluorouracil and leucovorin (FOLFOX4) was found to be more likely to produce a complete response than treatment with irinotecan plus fluorouracil and leucovorin (IFL) or irinotecan plus oxaliplatin (IROX). In addition, oxaliplatin regimens were more likely to result in successful surgical resections[26]. However, severe gastrointestinal toxicity and high mortality rates were observed with combination regimens containing daily bolus 5-FU/LV and oxaliplatin or irinotecan[27].

Irinotecan, a semisynthetic derivative of the natural alkaloid camptothecin, acts by inhibiting the action of topoisomerase I. Although in a previous study combination treatment with irinotecan plus weekly bolus IFL had proven superior to fluorouracil and leucovorin in patients with metastatic CRC[28], it did not result in a statistically significant improvement in either disease-free or overall survival in patients with stage Ⅲ colon cancer[29]. In a phase Ⅰ/Ⅱ study involving 23 patients with metastatic colorectal cancer, treatment with capecitabine plus oxaliplatin and irinotecan was well tolerated and the recommended daily dose of capecitabine was 1400 mg/m^2[30].

Capecitabine

Capecitabine, an oral prodrug of doxifluridine (prodrug of 5-FU), is absorbed through the gastrointestinal mucosa[18]. Oral capecitabine in combination with intravenous irinotecan was an active regimen in a phase Ⅱ study involving 65 patients with previously untreated metastatic colorectal cancer[31]. A Dutch Colorectal Cancer Group (DCCG) phase Ⅲ trial involving 820 patients with advanced colorectal cancer evaluated sequential versus combination chemotherapy with a fluoropyrimidine, irinotecan and oxaliplatin. In the DCCG trial, capecitabine plus irinotecan appeared to be a feasible first-line treatment; however, combination treatment did not significantly improve overall survival compared to the sequential use of cytotoxic drugs in advanced CRC[32,33]. In a Roswell Park Cancer Institute phase Ⅰ/Ⅱ study involving 25 patients with stage Ⅱ or Ⅲ rectal cancer, weekly intravenous oxaliplatin with daily oral capecitabine and radiotherapy was associated with a greater rate of pathological responses and demonstrated to be an effective neoadjuvant combination[34]. Capecitabine when administered in combination with perifosine showed promising clinical activity compared with single agent chemotherapy in a phase Ⅱ RCT involving 381 patients with previously untreated metastatic CRC[35]. Results of a phase Ⅱ study involving 146 patients with Stage T3 or T4 rectal cancer who received preoperative chemoradiotherapy with capecitabine plus oxaliplatin demonstrated significant clinical activity and acceptable toxicity[36]. This regimen is currently being evaluated in a phase Ⅲ randomized trial.

Ftorafur (tegafur) is a prodrug which is coadministered with an inhibitor of DPD (uracil). Coadministration allows for better bioavailability and uniform absorption[37]. In a RCT of 1608 patients, uracil/ftorafur (UFT) was associated with a higher convenience of care; thus, patients perceived adjuvant treatment with UFT plus leucovorin as more convenient than standard Ⅳ treatment with fluorouracil and leucovorin[38]. However, both therapies achieved similar disease-free and overall survival[39]. In the adjuvant treatment of 610 patients with stage Ⅲ colon or rectal cancer, postoperative treatment with UFT was successfully tolerated and improved relapse-free and overall survival in patients with rectal cancer; however, the expected benefits were not observed in colon cancer (HR = 0.89)[40]. In a phase Ⅱ RCT involving 58 elderly patients (range, 75 to 90 years) (range, 75 to 90 years) with measurable disease and no prior chemotherapy for metastatic disease, the UFT plus leucovorin regimen was moderately well tolerated and its activity was comparable to intravenous fluorouracil plus leucovorin, although there was increased GI toxicity in most patients[41,42].

Epidermal growth factor receptor inhibitors

Epidermal growth factor receptor (EGFR), a 170 kD transmembrane glycoprotein, is a member of the tyrosine kinase receptor family, ErbB. It is known to be overexpressed in malignancies of multiple tissues, including those of the colon, breast, lung and head and neck[43]. EGFR acts by affecting cell proliferation and survival and therefore has been known to contribute to metastatic progression[44]. Anti-EGFR therapies include monoclonal antibodies to EGFR and tyrosine kinase inhibitors.

In a multicenter phase Ⅱ trial of 74 patients with metastatic colorectal cancer, cetuximab seemed to positively interact with oxaliplatin and capecitabine[45]; however, its correct use in first-line treatment needs to be assessed in phase Ⅲ trials. In another phase Ⅱ study of 344 patients with metastatic colorectal cancer, cetuximab in combination with fluorouracil, leucovorin and oxaliplatin (FOLFOX4) demonstrated a higher overall response rate (46% *vs* 36%)[46] and significantly improved progression-free survival (HR = 0.567, P = 0.0064) compared to FOLFOX4 alone[47]. First line treatment with cetuximab plus fluorouracil, leucovorin and irinotecan was found to reduce the risk of metastatic progression in a Phase Ⅲ study of 1198 patients with epidermal growth factor receptor-positive colorectal cancer with unresectable metastases[48]. A significant increase in resectability was demonstrated by cetuximab in a phase Ⅱ study of

patients with non-resectable colorectal liver metastases when given in combination with FOLFOX6 or FOLFIRI as neoadjuvant chemotherapy[49]. Moreover, biweekly cetuximab plus irinotecan as second-line treatment has shown significant anti-tumor activity in patients with irinotecan-refractory metastatic CRC[50]. Panitumumab, a humanized monoclonal antibody to EGFR, when given in combination with fluorouracil, leucovorin and irinotecan as first-line treatment, has been well tolerated and showed promising activity in patients with metastatic colorectal cancer[51]. In another phase Ⅱ study, panitumumab monotherapy was found to be active in Japanese patients with chemotherapy-refractory metastatic CRC[52]. Immunogenicity of panitumumab when given in combination with oxaliplatin or irinotecan-based chemotherapy was found to be similar to the immunogenicity observed in the monotherapy setting in a phase Ⅲ study of patients with metastatic CRC[53].

Although the mechanism of action and safety profile of tyrosine kinase inhibitors such as gefitinib, sunitinib and erlotinib warrant further study in combination with standard regimens, early phase Ⅰ/Ⅱ studies showed promising activity and results suggest that they can be safely combined with standard regimens as first-line treatment[54-57].

Angiogenesis inhibitors

Another strategy to control cell proliferation in malignant tissues is the inhibition of new blood vessel formation. As of now, the main focus has been on inhibiting the protein that stimulates blood vessel proliferation, *i.e.*, the vascular endothelial growth factor (VEGF). The role of bevacizumab, a humanized monoclonal antibody against VEGF, is currently being studied in several randomized trials in the United States and Europe. Bevacizumab, when given in combination with oxaliplatin-based adjuvant therapy, did not prolong disease-free survival and demonstrated a detrimental effect in a phase Ⅲ study on patients with resected stage Ⅲ colon cancer[58]. Although an uncommon occurrence, use of bevacizumab in colorectal cancer has been shown to be associated with an increased risk of bowel perforation and fistula formation that occurs in a small proportion of CRC patients[59]; however, high dose bevacizumab when administered with IFL was well tolerated and regarded as a highly active regimen in patients with previously untreated CRC[60]. In a phase Ⅱ study in patients with previously untreated metastatic CRC, bevacizumab in combination with dose-reduced capecitabine and irinotecan was well tolerated and resulted in favorable outcomes[61]. In another randomized phase Ⅱ study of patients with previously untreated metastatic CRC receiving a fluorouracil-based chemotherapy regimen, addition of bevacizumab significantly increased overall and progression-free survival[62,63].

DISCUSSION

Although during the last decade substantial progress has been made in the diagnosis and successful treatment of colorectal cancer, clinicians and researchers still face challenges in the detection and management of the disease. Further clarification of the pathology of colorectal cancer at the molecular level may improve treatment options. The ultimate goal of scientists and clinicians in the field of cancer research is aimed not only at long-term survival of patients with this condition but also improvement of health-related quality of life. Pharmacological treatment of colorectal cancer has increased the rate of survival. While incorporation of new cytotoxic drugs and targeted agents has widened the treatment options for patients with metastatic colorectal cancer, combination regimens of standard chemotherapeutic drugs with newer agents have led to an increase in overall as well as progression-free survival. These newer combination regimens have contributed to increased rates of resectability in patients with potentially resectable tumors as well as improved health-related quality of life. Technology has improved the precision of radiation delivery to deep seated tumors. In order to gain the most benefit from these newer chemotherapeutic regimens and technologies, it is imperative to incorporate well-designed, multicenter studies with internationally standardized detection protocols in clinical trials with close collaboration between researchers and clinicians to cope with the vast quantity of data generated.

COMMENTS

Background

This review aims to explore the status of drug regimens, including synthetic drugs alone or in combination with biological agents, available for the treatment of colorectal cancer.

Research frontiers

Several new agents, both synthetic and biological, are currently being studied in clinical trials for their potential as part of the regular drug regimens for treatment of colorectal cancer.

Innovations and breakthroughs

After screening around 2300 randomized controlled trials, the authors found that the newer agents are well-tolerated and their addition to the standard chemotherapeutic drug regimens have led to an improvement in overall as well as progression-free survival in patients with metastatic colorectal cancer.

Applications

This review provides an update on the status of the synthetic drugs and treatment regimens available for the treatment of colorectal cancer.

Terminology

Fluoropyrimidines: Fluoropyrimidines are anti-metabolite agents widely used in the treatment of various cancers that act by inhibiting the enzyme thymidylate synthase. Angiogenesis: Angiogenesis is a physiological process of formation of new blood vessels from pre-existing vessels. EGFR: Epidermal growth factor receptor, a transmembrane glycoprotein, is a member of the tyrosine kinase receptor family, ErbB.

Peer review

This manuscript is a meta-analysis of current pharmacological treatments for colorectal cancer. The data presented are generally good and may be interesting for clinicians involved in this field.

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SYSTEMATIC REVIEW

Robotic surgery for rectal cancer: A systematic review of current practice

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Abstract

AIM: To give a comprehensive review of current literature on robotic rectal cancer surgery.

METHODS: A systematic review of current literature *via* PubMed and Embase search engines was performed to identify relevant articles from january 2007 to november 2013. The keywords used were: "robotic surgery", "surgical robotics", "laparoscopic computer-assisted surgery", "colectomy" and "rectal resection".

RESULTS: After the initial screen of 380 articles, 20 papers were selected for review. A total of 1062 patients (male 64.0%) with a mean age of 61.1 years and body mass index of 24.9 kg/m^2 were included in the review. Out of 1062 robotic-assisted operations, 831 (78.2%) anterior and low anterior resections, 132 (12.4%) intersphincteric resection with coloanal anastomosis, 98 (9.3%) abdominoperineal resections and 1 (0.1%) Hartmann's operation were included in the review. Robotic rectal surgery was associated with longer operative time but with comparable oncological results and anastomotic leak rate when compared with laparoscopic rectal surgery.

CONCLUSION: Robotic colorectal surgery has continued to evolve to its current state with promising results; feasible surgical option with low conversion rate and comparable short-term oncological results. The challenges faced with robotic surgery are for more high quality studies to justify its cost.

Key words: Rectal cancer; Robotics; Minimal invasive surgery; Systematic review; Rectal surgery

Core tip: This systematic review summarizes current evidence on the role of robotic surgery for the treatment of rectal cancer. It is a timely article as minimal invasive surgery has proven to benefit patients with colonic cancers but conventional laparoscopic surgery for the treatment for rectal cancer remains controversial due to its steep learning curve. Robotic-assisted surgery has technological advances, which may have the potential to overcome some of the limitations of conventional laparoscopic surgery.

Mak TWC, Lee JFY, Futaba K, Hon SSF, Ngo DKY, Ng SSM. Robotic surgery for rectal cancer: A systematic review of current practice. *World J Gastrointest Oncol* 2014; 6(6): 184-193 Available from: URL: http://www.wjgnet.com/1948-5204/full/v6/i6/184.htm DOI: http://dx.doi.org/10.4251/wjgo.v6.i6.184

INTRODUCTION

Minimally invasive surgery over the past two decades has revolutionised surgical management of colorectal cancers. Despite its initial scepticism, various randomised controlled trials have now demonstrated its short-term and long-term benefits over conventional open surgery in the treatment of colonic cancer such as faster recovery, decreased morbidity and reduced hospital length of stay with comparable oncological result and survival outcome[1-4]. However, laparoscopic colorectal surgery has limitations. These concerns were high-lighted not only by the high conversion rate but also the initially high proportion of circumferential resection margin (CRM) positive rates in the medical research council colorectal cancer (MRC-CLASICC) trial for laparoscopic rectal surgery[5]. The ability to perform total mesorectal excision (TME) laparoscopically requires intensive training. Limitations of conventional laparoscopic surgery include: 2-dimension view, unstable assistant controlled camera, poor ergonomics, straight tip instruments, fulcrum effect and enhanced tremor effect.

Various attempts have been made to seek alternative techniques to overcome some of these limitations. For example, single incision laparoscopic surgery has reduced the number of incisions and ports required for minimal invasive colonic surgery producing a better cosmetic result and reduction in wound pain[6]. Natural orifice translumenal endoscopic surgery (NOTES) aims to eliminate external incision by gaining access using the transvaginal, transgastric, transvesical and transrectal approach, which has been shown to be feasible on animal models[7-9]. However, there are still many hurdles in NOTES (*e.g.*, determining a safe access into the peritoneal cavity, developing a reliable method on the closure of viscotomy, minimising the infection and tumour seedling risk, developing a stable and versatile platform for suturing, managing complications from NOTES and training issues), which need to be addressed before its routine application on Human subjects.

The da Vinci® robot is the first robotic surgical system approved by the United States Food and Drug Administration in 2000. It has evolved from its first generation robot in 1999, the da Vinci standard®, to the current third generation da Vinci-Si HD®, which was launched in 2009. The da Vinci Si-HD® has features such as: (1) dual operating console capability for combined operating and training; (2) enhanced operator-controlled 3D high-definition vision; (3) endowrist™ technology allowing 7 degrees of freedom intra-abdominally; and (4) tremor elimination with improved dexterity. Weber *et al*[10] and Hashizume *et al*[11] first performed colorectal robotics surgery in 2002[10,11]. Prior to this, robotic surgery was already successfully performed on cardiothoracic, urological and general surgical[12-14] patients.

Robotic rectal surgery has potential advantages over conventional laparoscopic rectal surgery: Surgeon motion filter for tremor-free surgery, high definition three-dimensional images, surgeon control camera on a stable platform and increased degree of freedom of the operating instruments. The master and slave system allows improved ergonomics for the surgeon. As the surgical field mainly confines to the pelvic cavity, it allows a stable platform for precision surgery to be performed in a confined space. For the above reasons, robotic technology may be more suitable and may translate more benefits when used for rectal cancers than colonic cancers.

Several review articles have attempted to summarize up-to-date practice and results of robotic colorectal surgery. However some studies included data from both robotic colonic and rectal resections, which may not give a focused overview of the benefits and risks of robotic rectal surgery[15-17]. Other studies included more than one study from the same institute with overlapping period of assessment, which may cause duplication of results[15,18]. Although meta-analysis of robotic rectal resection have been published, studies included were from non-randomised studies[19,20]. Hence we feel that an up-to-date systematic review on robotic rectal surgery is most appropriate and warranted.

This article aims to compare robotic-assisted rectal surgery with conventional laparoscopic rectal surgery for patients with rectal cancers. The current status of robotic rectal surgery focusing on its efficacy, feasibility and oncological safety will also be discussed.

MATERIALS AND METHODS

Two reviewers independently (T.M. and K.F.) performed a literature search *via* PubMed, Google Scholar, Cochrane Library and Embase database during the period between January 2007 to November 2013. Search terms such as "robotic surgery", "surgical robotics", "laparoscopic computer-assisted surgery" and "rectal resection" were used. Only english language published studies were considered. In addition, the reference lists of selected articles were searched manually. Abstract publications from conferences were excluded from this review. Published data from robotic rectal surgery using the Da Vinci® Surgical System (Intuitive Surgical, Mountain View, Sunnyvale, CA, United States) were only included in order to reduce clinical heterogeneity and the authors recognise that currently it is the only operating system available.

Inclusion criteria for search include randomised and non-randomised controlled trials, comparison studies, case series and case report. The target population consists of patients aged > 18 years with histologically proven rectal cancers.

This systematic review was conducted according to a guidance from the Centre for Reviews and Dissemination[21] and the Cochrane Handbook[22]. The review is reported in line with the Preferred Reporting Items for Systematic Reviews and Meta-analyses statement[23]. Selected articles were screened independently by two reviewers for bias using The Cochrane Collaboration's tool for assessing risk of bias[22].

Two reviewers (T.M and K.F.) extracted data from the manuscripts of selected articles including the study de-

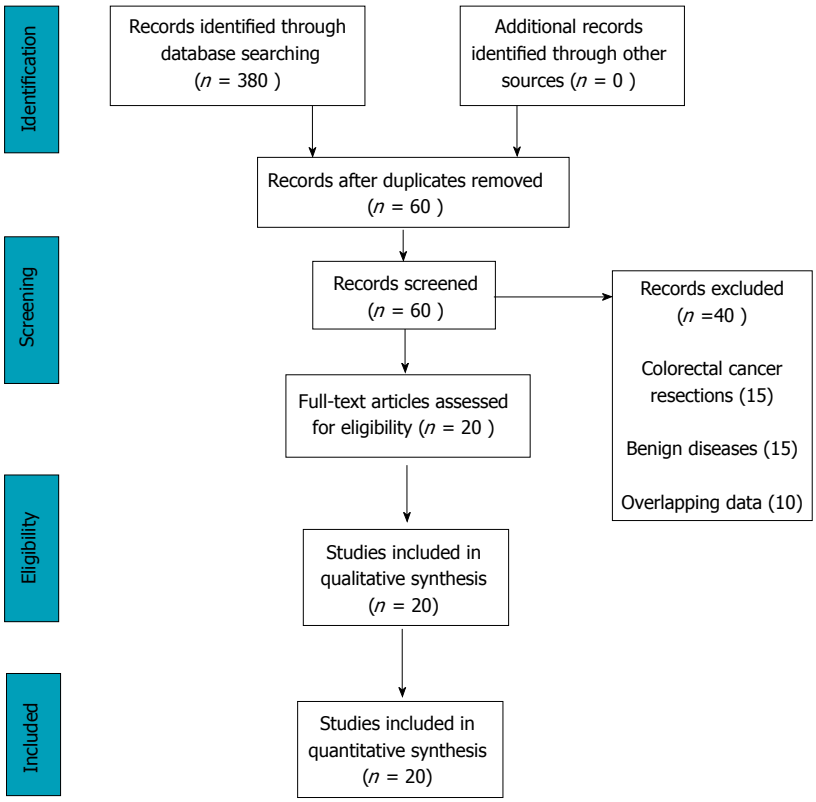

Figure 1 Systematic review Prisma flow diagram.

sign, patient demographics, clinical characteristics, site of malignancy, types of intervention, peri-operative details, pathological results, and post-operative outcomes.

RESULTS

After the initial screen of 380 articles, 60 articles met the predefined inclusion criteria. 15 articles with inseparable data from colonic cancers, 15 articles with benign colorectal disease and 10 articles from the same institutes with overlapping study period were excluded to avoid duplication. 20 studies were selected for review, which comprised of: 13 comparison studies and 7 case series (Figure 1). A large proportion of these studies came from South Korea[24-31] (40.0%) followed by United States[32-36] (25.0%), Italy[37-39] (15.0%), Singapore[40,41] (10.0%) and Turkey[42] (5.0%) and Romania[43] (5.0%) (Table 1).

Surgical technique

There are generally two recognised techniques for Robotic Rectal surgery; the hybrid technique or the total robotic technique. The hybrid technique involves a combination of laparoscopic and robotic techniques to be used in different stages of the operation. The advantage of this method allows a shorter operative time, in particular for rectal cancer operation where the left colon and splenic flexure are mobilised by conventional laparoscopic technique followed by the robotic pelvic dissection[24,27,31,33,34,36-38,40]. Total robotic technique allows the entire operation to the carried out robotically which can either be *via*: (1) single docking technique- which only requires one docking of the robotic cart with repositioning of the robotic arms according to the operative field[25,26,28,39,41,42]; or (2) dual docking technique which requires the operating table to be positioned twice to the desired operative field[30]. Amongst the selected articles, there was 8 Hybrid, 7 Total robotic, 4 combinations of hybrid and total robotic and 1 reverse-hybrid techniques. Study from Park *et al*[35] reported a reverse-hybrid whereby robotic lymphovascular (inferior mesenteric artery) and pelvic dissection is performed before laparoscopic mobilisation of left colon and splenic flexure mobilisation.

Clinical outcomes

Patient demographics: A total of 1062 patients were included in the study. The mean age was 61.1 years and 64.0% were male. The average Body mass index BMI was 24.9 kg/m^2. Out of 1062 robotic-assisted operations, there were 831 (78.2%) anterior and low anterior resections, 132 (12.4%) intersphincteric resection with coloanal anastomosis, 98 (9.3%) abdominoperineal resections and 1 (0.1%) Hartmann's operation.

Operative procedures: The review identified 1062 and 706 robotic and laparoscopic rectal operations respectively (Table 2). Mean operation time in the robotic group was 281.8 min (range, 180.0-528.0) compared with the laparoscopic group 242.6 min (range, 158.1-344.0). 7 out of the 11 comparison studies found robotic rectal surgery to have a significantly longer operative time when

Table 1 Characteristics of studies on robotic rectal surgery

Ref.	Country	Year	Study type	No. of robotic patients	Gender M:F	Mean age (yr)	BMI (kg/m^2)	Robotic Technique	Type of operation			
									AR/ LAR	ISR	APR	Hartmann's operation
Baik *et al*[24]	South Korea	2009	Comparison	56	37:19	60.0	23.4	Hybrid	56	-	-	-
Ng *et al*[40]	Singapore	2009	Case Series	8	5:3	55.0[1]	-	Hybrid	8	-	-	-
Patriti *et al*[37]	Italy	2009	Comparison	29	11:18	68.0	24.0	Hybrid	19	5	5	-
Bianchi *et al*[38]	Italy	2010	Comparison	25	18:7	69.0	24.6	Total/hybrid	18	-	7	-
Pigazzi *et al*[32]	United States, Italy	2010	Case Series	143	87:56	62.0	26.5	Total/hybrid	80	32	31	-
Zimmern *et al*[33]	United States	2010	Case Series	58	34:24	60.9	27.5	Hybrid	47	-	11	-
Baek *et al*[34]	United States	2011	Comparison	41	25:16	63.6	25.7	Hybrid	33	2	6	-
Koh *et al*[41]	Singapore	2011	Case Series	20	13:8	61.0	23.8	Total	19	-	1	-
Kwak *et al*[25]	South Korea	2011	Comparison	59	39:20	60.0[1]	23.3	Total	54	5	-	-
Leong *et al*[26]	South Korea	2011	Case Series	29	23:6	61.5[1]	23.3	Total	-	29	-	-
Park *et al*[27]	South Korea	2011	Comparison	52	28:24	57.3	23.7	Hybrid	52	-	-	-
Kim *et al*[28]	South Korea	2012	Comparison	100	71:29	57.0	24.0	Total	100	-	-	-
Park *et al*[35]	United States	2012	Case Series	30	16:14	58.0[1]	27.6	Reverse-hybrid	5	19	6	-
Shin *et al*[29]	South Korea	2012	Comparison	17	-	-	-	Total/hybrid	17	-	-	-
Erguner *et al*[42]	Turkey	2013	Comparison	27	14:13	54.0	28.3	Total	27	-	-	-
Kang *et al*[30]	South Korea	2013	Comparison	165	104:61	61.2	23.1	Total	164	-	-	1
Park *et al*[31]	South Korea	2013	Comparison	40	28:12	57.3	23.9	Hybrid	-	40	-	-
Stanciulea *et al*[43]	Romania	2013	Case Series	100	66:34	62.0	26.0	Total/Hybrid	77	-	23	-
D'Annibale *et al*[39]	Italy	2013	Comparison	50	30:20	66.0	-	Total	50[2]	-	-	-
Fernandez *et al*[36]	United States	2013	Comparison	13	13:0	67.9	-	Hybrid	5	-	8	-
Total				1062	680:382	61.1	24.9		831	132	98	1

[1]median value; [2]TME: Paper did not specify operation. AR: Anterior resection; LAR: Low anterior resection; ISR: Intersphincteric resection; APR: Abdominoperineal resection.

Table 2 Perioperative and postoperative outcomes

Ref.	No. of patients		Conversion (%)		Mean OR time (min)		Blood loss (mL)		Overall post-op morbidity (%)		Anastomotic leak (%)		Erectile dysfunction (%)		Voiding dysfunction (%)		LOS (d)	
	Rob	Lap	Rob	Lap	Rob	Lap	Rob	Lap	Rob	Lap	Rob	Lap	Rob	Lap	Rob	Lap	Rob	Lap
Baik *et al*[24]	56	57	0	10.5	190.1	191.1	-	-	10.7	19.3	1.8	7.0	-	-	-	-	5.7	7.6
Ng *et al*[40]	8	NA	0	NA	193.8	NA	min	NA	12.5	NA	0	NA	-	-	-	-	5.0	NA
Patriti *et al*[37]	29	37	0	18.9	202.0	208.0	137.0	127.0	26.0	32.8	6.8	2.7	5.5	16.6	-	-	11.9	9.6
Bianchi *et al*[38]	25	25	0	4.0	240.0	237.0	-	-	16.0	24.0	4.0	8.0	-	-	-	-	6.5	6.0
Pigazzi *et al*[32]	143	NA	4.7	NA	297.0	NA	min	NA	41.3	NA	10.5	NA	-	-	-	-	8.3	NA
Zimmern *et al*[33]	58	NA	3.7	NA	338.0	NA	232.0	NA	25.9	NA	3.4	NA	-	-	-	-	6.0	NA
Baek *et al*[34]	41	41	7.3	22.0	296.0	315.0	-	-	22.0	26.8	7.3	2.4	-	-	-	-	6.5	6.6
Koh *et al*[41]	20	NA	0	NA	306.0	NA	-	-	23.8	NA	0	NA	-	-	-	-	6.4	NA
Kwak *et al*[25]	59	60	0	3.4	270.0	228.0	-	-	32.2	26.7	13.6	10.2	-	-	-	-	-	-
Leong *et al*[26]	29	NA	0	NA	325.0	NA	-	-	37.9	NA	10.3	NA	-	-	-	-	9.0[1]	NA
Park *et al*[27]	52	123	0	0	232.6	158.1	-	-	19.2	12.2	9.6	5.6	-	-	0	1.6	10.4	9.8
Kim *et al*[28]	100	NA	0	NA	188.0	NA	-	-	11.0	NA	2.0	NA	36.6	NA	6.0	NA	7.1	NA
Park *et al*[35]	30	NA	0	NA	369.0	NA	100.0	NA	36.7	NA	4.2	NA	0	NA	0	NA	4.0[1]	NA
Shin *et al*[29]	17	12	0	1.0	396.5	298.8	188.8	229.2	16.7[2]	20.0[2]	0	0	-	-	1.0	2.0	10.7	9.6
Erguner *et al*[42]	27	37	0	0	280.0	190.0	50.0	125.0	11.1	21.6	0	8.1	0	2.7	-	-	4.0	5.0
Kang *et al*[30]	165	165	0.6	1.8	309.7	277.8	133.0	140.1	20.6	27.9	7.3	10.8	-	-	2.4	4.2	10.8	13.5
Park *et al*[31]	40	40	0	0	225.0	183.7	45.7	59.2	15.0	12.5	7.5	5.0	A	A	A	A	10.6	11.3
Stanciulea *et al*[43]	100	NA	4.0	NA	180.0[1]	NA	150.0[1]	NA	30.0	NA	9.0	NA	3.8	NA	7.7	NA	10.0[1]	NA
D'Annibale *et al*[39]	50	50	0	12.0	270.0[1]	280.0[1]	-	-	10.0	22.0	10.0	22.0	5.6	56.5	A	A	8.0[1]	10.0[1]
Fernandez *et al*[36]	13	59	8.0	17.0	528.0[1]	344.0	157.0[1]	200.0	-	-	20.0	7.0	-	-	-	-	13.0[1]	8.0[1]

[1]Median; [2]Overall figures for colorectal resections (not just rectal). OR: Operating room; LOS: Length of stay; A: Erectile and voiding dysfunction was assessed and scored with the International Index of Erectile Function score and/or the International Prostate Symptom score respectively; NA: Not available.

compared to the laparoscopic surgery[25,27,29-31,36,42]. The remaining 4 studies found laparoscopic rectal surgery to be longer but none were statistically significant[24,34,37,39]. Most authors identified the longer time taken with robotic surgery to be due to docking and changing of the robotic arms.

Conversion rates for the robotic group ranges from 0% to 8.0% compared to 1.8% to 22% in the laparoscopic group. Both groups cited reasons for conversion such as obesity, difficulty anatomy, bulky tumour, narrow pelvis, adhesions from previous surgery, equipment malfunction and intra-operative complications (*e.g.*, massive bleeding, rectal perforation). In 10 comparison studies, there were no conversions in the robotic group when

Table 3 Oncological outcomes

Ref.	No. of patients		Mean follow-up (mths)		NeoCRT (%)		Lymph nodes harvested (mean)		TME grade complete (%)		CRM +ve (%)		DRM (cm)		Robotic Recurrence (%)	3 yr Robotic Survival (%)	
	Rob	Lap	Rob	Lap	Rob	Lap	Rob	Lap	Rob	Lap	Rob	Lap	Rob	Lap		DS	OS
Baik *et al*[24]	56	57	14.3 (both)		8.9	12.2	18.4	18.7	92.9	75.4	7.1	8.8	4.0	3.6	-	-	7.6
Ng *et al*[40]	8	NA	1.5	NA	-	-	12.9	NA	-	-	0	NA	> 2.0	NA	-	-	NA
Patriti *et al*[37]	29	37	29.2	18.7	24.1	5.4	10.3	11.2	-	-	0	0	2.1	4.5	None	100.0	9.6
Bianchi *et al*[38]	25	25	10.0 (both)		52.0	40.0	19.7	18.2	-	-	0	4.0	2.0	2.0	None	-	6.0
Pigazzi *et al*[32]	143	NA	17.4	NA	65.1	-	14.1	NA	-	-	0.7	NA	2.9	NA	1.5	77.6	NA
Zimmern *et al*[33]	58	NA	13.2	NA	39.7	NA	14.1	NA	-	-	0	NA	-	-	5.2	-	NA
Baek *et al*[34]	41	41	-	-	80.5	43.9	13.1	16.2	-	-	2.4	4.9	3.6	3.8	-	-	6.6
Koh *et al*[41]	20	NA	-	-	9.5	NA	17.8	NA	-	-	5.3	-	3.7	-	-	-	NA
Kwak *et al*[25]	59	60	17.0	13.0	13.6	8.5	20.0	21.0	-	-	1.7	0	-	-	-	-	-
Leong *et al*[26]	29	NA	-	-	37.9	NA	16.0	NA	-	-	7.0	NA	0.8	NA	-	-	NA
Park *et al*[27]	52	123	-	-	23.1	8.1	19.4	15.9	-	-	1.9	2.4	2.8	3.2	-	-	9.8
Kim *et al*[28]	100	NA	24.0	NA	32.0	NA	20.0	NA	-	-	1.0	NA	2.7	NA	-	-	NA
Park *et al*[35]	30	NA	-	-	66.7	NA	20.0	NA	83.3	NA	0	NA	-	-	-	-	NA
Shin *et al*[29]	17	12	-	-	-	-	18.4[2]	15.9[2]	-	-	-	-	-	-	-	-	9.6
Erguner *et al*[42]	27	37	-	-	14.8	21.6	16.0	16.0	100.0	70.6	0	0	4.0	4.0	-	-	5.0
Kang *et al*[30]	165	165	22.4[1] (both)		23.6	21.8	15.0	15.6	-	-	4.2	6.7	1.9	2.0	-	-	-
Park *et al*[31]	40	40	6.0	6.0	80.0	50	12.9	13.3	-	-	7.5	5.0	1.4	1.3	-	-	-
Stanciulea *et al*[43]	100	NA	24.0[1]	NA	58.0	NA	14.0[1]	NA	-	-	1.0	-	3.0	-	2.0	NA	90.0
D'Annibale *et al*[39]	50	50	12.0	12.0	68.0	56.0	16.5	13.8	-	-	0	0	3.0	3.0	-	-	-
Fernandez *et al*[36]	13	59	-	-	77.0	54.0	16.0	20.0	69.0	73.0	0	2.0	-	-	-	-	-

[1]Median; [2]Overall figures for colorectal resections (not just rectal). Rob: Robotic-assisted surgery; Lap: Conventional laparoscopic surgery; NeoCRT: Neo-adjuvant chemoradiotherapy; TME: Total mesorectal excision; CRM: Circumferential resection margin; DRM: Distal resection margin; DS: Disease free survival; NA: Not available.

compared to the laparoscopic group[24,25,27-29,31,37-39,42].

Intraoperative blood loss was compared in 6 studies in this review[29-31,36,37,42]. Five studies found the laparoscopic group had more blood loss when compared to the robotic group but only two of these studies were found to be statistically significant[29,42].

Post-operative outcome

The overall post-operative morbidity in both groups was found to be similar with median of 20.0% (range 10.7%-41.3%) in the robotic group compared with 22.3% (range 12.2%-32.8%) in the laparoscopic group (Table 2). These include anastomotic leak, chest infection, urinary tract infection, postoperative ileus, urinary retention, DVT, wound dehiscence and intra-abdominal collection. Anastomotic leak was also assessed separately as it carries a significant morbidity and mortality. It has been postulated that with the advanced technology, robotic assisted surgery may reduce its incidence with better operative vision and a more precise dissection technique. In this review, median anastomotic leak rate was found to be similar with mean of 6.4% (range, 0%-20.0%) in robotic group compared to 7.4% (range, 0%-22.0%) in laparoscopic group. Preservation of the pelvic autonomic nerves during pelvic surgery is important in order to prevent erectile and voiding dysfunctions. In this review, 7 studies[28,31,35,37,39,42,43] assessed erectile dysfunction and found the incidence of complication ranged from 0% to 36.6% in the robotic group compared to 2.7% to 56.5% in the laparoscopic group. Four of these papers were comparative studies, where Patriti *et al*[37] found a higher proportion of erectile dysfunction in the laparoscopic group (16.6% *vs* 5.5% respectively) but this was not significant. Two papers reported sexual and voiding function using the International Index of Erectile Function score (IIEF-5) and the International Prostate Symptom score respectively[31,39]. In the study by Park *et al*[31], patients were asked to complete the questionnaires preoperatively, 3 and 6 mo postoperatively. In terms of erectile dysfunction, the laparoscopic group had a significantly higher incidence than the robotic group. The robotic group also shown a faster rate of improvement when assessed at 3 and 6 mo. However there was no difference found in terms of voiding function. D'Annibale *et al*[39] reported 1-year follow-up assessment of erectile dysfunction and found a significant proportion of sexually active patients in the laparoscopic group (13 out of 23; 56.5%) reported erectile dysfunction when compared with the robotic group (1 out of 17; 5.6%). However this result may need to be interpreted with caution as there were a high non-participation rate in the 30 patients selected in each group (laparoscopic group = 23.3% *vs* robotic assisted group = 40.0%).

Length of stay found the median stay of 7.1 d (range 4-13.0 d) in the robotic procedures compared with median of 9.6 d (range 5-13.5 d) performed by the laparoscopic procedures. Only 2 out of 11 studies showed significantly shorter hospital stay in the robotic group[24,30].

Oncological outcome

Robotic rectal surgery achieved comparable results with laparoscopic surgery in terms of percentage of CRM positivity, mean distal resection margin (Table 3). All studies documented that rectal cancer patients who

Table 4 Cost of Robotic rectal surgery

Ref.	Country	Year	Study type	No. of rectal cancer patients			Average total hospitalisation cost (United States $)			*P* value
				Robotic	Laparoscopic	Open	Robotic	Laparoscopic	Open	
Baik *et al*[24]	United States	2011	Comparison	41	41	-	83915	62601	-	0.092
Kwak *et al*[25]	South Korea	2011	Comparison	59	59	-	Robotic x3 Laparoscopic cost		NA	NA
Leong *et al*[26]	South Korea	2011	Case Series	29	-	-	Robotic x3 Laparoscopic cost		-	-
Kim *et al*[28]	South Korea	2012	Comparison	100	-	100	12-15000	5000	-	-

Rob: Robotic-assisted surgery; Lap: Conventional laparoscopic surgery; NA: Not available.

were preoperatively diagnosed to have T3 or T4 tumour +/- lymph node invasion were given neoadjuvant chemoradiotherapy. Percentage of patients who received neoadjuvant chemoradiotherapy was documented in 11 comparative studies, varying from 8.9% to 80.5% in the robotic group compared with 5.4% to 56.0% in the laparoscopic group[24,25,30,31,34,36-39,42]. The quality of the TME was also assessed. Two studies comparing TME quality after robotic and laparoscopic dissection found the former to be significantly superior[24,42] whereas the study by Fernandez *et al*[36] found the laparoscopic group to be superior but this was not statistically significant. The studies showed there was minimal difference between the number of lymph nodes retrieved with robotic assisted (range, 10.3 to 20.0) and laparoscopic rectal resection (range, 11.2 to 21). Recurrence of cancer from 6 studies ranged from no recorded recurrence to 5.5%. In a study by Kwak *et al*[25], there were no significant differences found between the robotic-assisted group and laparoscopy assisted group in terms of loco-regional recurrence, distant metastasis and total recurrence. Three-year disease free survival ranges from 77.6% to 100% with overall survival between 90% to 97%. The study by Kang *et al*[30] found no difference in 2-year survival between robotic assisted group (83.5%), laparoscopy group (81.9%) and open surgery (79.7%) ($P = 0.855$).

Learning curve

Within the selected articles, there were only 3 papers which looked into learning curve for robotic rectal surgery[31,32,39]. Pigazzi *et al*[32] found operative time decreased significantly after 20 cases. With intersphincteric resections, Park *et al*[31] found the learning curve plateau after 17 cases by using the moving average method. In one paper the author's opinion was that the numbers of cases require for learning can be as low as two cases if performed by an already skilled laparoscopic surgeon[38]. D'Annibale *et al*[39] found mean operative time decreased from 312.5 min in the first 25 procedures to 238.2 min in the last 10 procedures ($P = 0.002$). Following cusum analysis, this study showed that learning curve in robot group was achieved after 22 cases[39].

Cost

A review of the selected articles found four studies, which looked into the cost of robotic surgery (Table 4). In two of the studies, the cost of robotic rectal surgery was estimated to be three times more expensive than laparoscopic rectal surgery[25,26]. The remaining two studies found also robotic rectal surgery to be more expensive when compared to laparoscopic and open rectal surgery but the figures in these studies did not show statistical significance[28,34]. Authors also highlighted the fact that the provision of health is different between countries such as in South Korea.

DISCUSSION

This systematic review suggests robotic-assisted surgery to be feasible and safe. We have selected 20 articles for review out of 380 articles, which met our selection criteria. We deliberately set the inclusion period to be within the past 6 years as it will exclude small case series where authors may not have attained the desired learning curve and also a more recent data-set may give a more accurate reflection of the current practice and capability of the da Vinci robotic systems.

Previous systematic reviews have reported similar outcomes to our study[15,16,18]. They concluded robotic-assisted rectal surgery to be feasible and safe. Similar to our review, conversion rates tend to be lower in the robotic-assisted group when compared to the laparoscopic group. This may have important implications as converted cases are associated with greater morbidity and tumour recurrence[3]. Many authors identified lower conversion rates in the robotic group to be associated with superior visualisation, better exposure and endowrist™ technology.

In our review we found overall complication rates between robotic and laparoscopic group to be similar. These perceived advantages also did not translate to lower anastomotic leaks in the robotic group, which may be due to the fact that the aetiology for anastomotic leak is multifactorial (*e.g.*, patient nutrition, underlying comorbidity, neoadjuvant chemoradiotherapy, surgical technique, blood supply, tension to anastomosis, *etc.*) and therefore an adequately powered study is required. Intraoperative blood loss only resulted in two studies, which found laparoscopic group to have a statistically greater blood loss than the robotic group[29,42].

The short-term oncological outcome using conventional surgical yardsticks for rectal cancer dissection seems to be comparable between the two groups. CRM and distal resection margins are comparable to laparoscopic group. Quality of the TME dissection is important as breach of the TME envelope may increase local and distant recurrence. In this review, only three studies as-

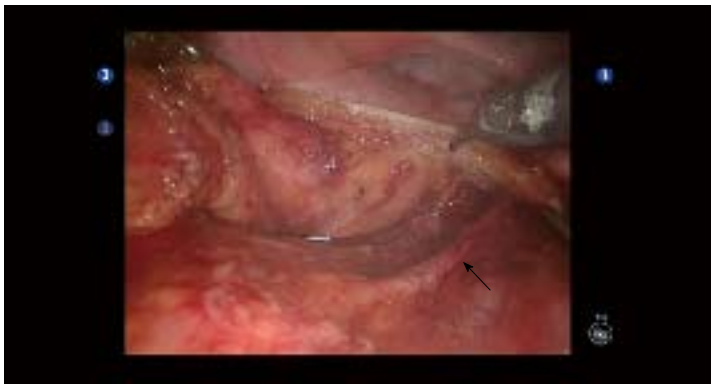

Figure 2 Robotic pelvic dissection. High definition 3-D view of the pelvis with the right hypogastric nerve (arrow) identified and protected.

sessed the quality of the TME specimen macroscopically with two comparative studies found robotic dissection to be superior. With emerging data favouring TME *via* minimal invasive approach over open surgery[5,44], robotic surgery may offer additional advantage.

Traditionally long operative times are related with increased morbidity, which is likely to be related to the difficulty of the operation[45]. Robotic surgery has been found to have a longer operative time when compared to laparoscopic or open rectal surgery. Attempts have been made to reduce robotic operating time by adopting the hybrid approach. However this will require the surgeon to be skilled at both robotic as well as conventional laparoscopic surgery. Also the perceived advantage of robotic surgery may be lost during inferior mesenteric artery dissection, which may increase the chance of nerve damage as well as additional cost of laparoscopic instruments. Prolonged operative times are most likely to be related to technical aspects of the operation (time taken to dock and redock the robot as well as changing of robotic arms) rather than the operative difficulty. Indeed the overall complication rates between the robotic and the laparoscopic groups have been shown to be similar in this review, which further supports the theory that longer robotic operative time may not necessarily increase operative morbidity.

Cost of robotic surgery remained to be an important issue. Most papers identified the cost of the robot to be around United States $1.65 to 2 million, disposable robotic instruments costing United States $2000 each as well as the yearly maintenance cost United States $150000[24]. In this review article, it was not possible to include cost-effectiveness analysis studies. Baek *et al*[34] highlighted the fact that caution needs to be taken when interpreting costs as it may differ significantly between hospitals. Different healthcare system between countries will also have an impact on costs. However, maximising the use of the robot by different surgical specialties within the hospital might make savings to the overall running costs.

Identification and preservation of the pelvic autonomic nerves may be better with robotic surgery due to high definition 3-D image, tremor free surgery, surgeon operated camera platform and endowrist™ technology. Common sites of potential pelvic nerve damage leading to sexual dysfunction are: (1) superior hypogastric plexus, leading to ejaculation dysfunction on male patients and impaired lubrication in females; and (2) pelvic splanchnic nerves or the pelvic plexus- leading to erectile dysfunction in men. These perceived advantages may translate to decreased incidence of erectile dysfunction in male patients and urinary dysfunction as the CLASICC trial reported a 41% sexual dysfunction in men after laparoscopic rectal surgery when compared with 23% in the open rectal surgery group[46] (Figure 2). However, in this review although there were some encouraging results to suggest that robotic-assisted surgery is superior to conventional laparoscopic surgery in preventing sexual or urinary dysfunction, the evidence is not entirely clear due to high non-participation rates and possible type II error. Kim *et al*[47] also reported similar results where although the robotic-assisted group reported earlier recovery of erectile, sexual desire and urinary function when compared with the laparoscopic group, there was no difference in long-term follow-up.

In this review, we were unable to draw strong conclusion on the learning curve required for robotic surgery. However the range of 17-25 cases of robotic-assisted rectal surgery from experienced surgeons skilled at both open and laparoscopic surgery are quoted as the number required to achieve competency. The cases selected were very heterogeneous; only few studies used recognised method on assessing learning curve and one of studies were from expert's comment.

Although the da Vinci® robotic platform has produced promising results with at least comparable benefits to laparoscopic colorectal surgery, good quality studies are still required to demonstrate its benefits. The ROLARR (RObotic versus LAparoscopic Resection for Rectal cancer) study is a multicentre international randomised control trial with the primary aim to assess technical ease of robotic rectal operations. The secondary aims are to assess the quality of life, cost-effectiveness analysis and oncological outcome on disease-free and overall survival and local recurrence at 3-year follow-up. The study began recruiting in february 2011 and therefore results will not be available for sometime[48]. Other Robotic rectal surgical clinical trials currently registered on www.clinicaltrials.gov include centres from South Korea[49,50], China[51] and Hong Kong[52].

In summary, from this systematic review, in the au-

thors' opinion we can draw conclusions on the following: (1) robotic-assisted rectal surgery is feasible and safe; (2) it has a lower conversion rate when compared to laparoscopic group; (3) intra-operative blood loss resulted significantly less in the robotic group in 2 of the comparison studies; (4) postoperative morbidity and long-term voiding and sexual functions remain similar in both groups; (5) quality of the TME dissection is significantly better in some studies but nevertheless there were no significant differences found in short-term of oncological outcomes in both groups; and (6) robotic-assisted is more expensive than laparoscopic surgery. Hence the current challenges will be to justify the benefits of robotic rectal surgery over high costs.

COMMENTS

Background

The incidence of rectal cancers is increasing owing to the elderly population, westernised lifestyle and other environmental factors. Prognosis in rectal cancer can be related to the quality of surgery such as mesorectal integrity, margin status, and adequate lymph node dissection. Laparoscopic has been proven to reduce hospital stay, less pain and less bleeding but its role in rectal cancer surgery remains controversial due to its steep learning-curve. Da Vinci robotic-assisted rectal cancer surgery may be an effective tool but its effectiveness over laparoscopic surgery is unclear.

Research frontiers

Robotic-assisted rectal cancer surgery has technical advantages over conventional laparoscopic method such as tremor free surgery, high definition 3-D vision, stable platform and surgeon-control camera. These technological advances seem to be ideally suited for rectal cancer surgery as it may minimize inadvertent pelvic neurovascular injury and achieve good oncological results.

Innovations and breakthroughs

Conventional laparoscopic rectal surgery has been known to have a steep learning curve owing to 2-Dimensional view, assistant navigated camera and instruments with limited freedom of movement. Robotic-assisted rectal surgery has overcome some of these limitations with 3-Dimensional view, stable platform, surgeon-controlled camera and tremor-free surgery. However further high quality research is required see whether these advances can be translated to benefit patient care.

Applications

Readers will be able to have an unbiased view on the pros and cons of robotic-assisted rectal surgery. This systematic review has identified current evidence is based on case series and comparative reports and that has demonstrated robotic-assisted rectal surgery is feasible and safe. However as these studies demonstrated potential benefits of robotic surgery are not yet proven and that whether the high cost justify these benefits is still under debate.

Terminology

Laparoscopic surgery and robotic-assisted surgery are a form of minimal invasive surgery which has advantages over open operations such as less blood loss, faster recovery, less complications and better cosmetic results.

Peer review

This manuscript is an interesting and well done systematic review on robotic rectal surgery. Authors reported data according to the Prisma guidelines for systematic reviews and meta-analyses. This paper deserves publication.

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REVIEW

Genotypic characteristics of resistant tumors to pre-operative ionizing radiation in rectal cancer

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Abstract

Due to a wide range of clinical response in patients undergoing neo-adjuvant chemoradiation for rectal cancer it is essential to understand molecular factors that lead to the broad response observed in patients receiving the same form of treatment. Despite extensive research in this field, the exact mechanisms still remain elusive. Data raging from DNA-repair to specific molecules leading to cell survival as well as resistance to apoptosis have been investigated. Individually, or in combination, there is no single pathway that has become clinically applicable to date. In the following review, we describe the current status of various pathways that might lead to resistance to the therapeutic applications of ionizing radiation in rectal cancer.

Key words: Ionizing radiation; DNA double-strand break; Non-homologous end-joining pathway; DNA-PKcs; Ku proteins; Complete pathological response; Radiation therapy; Apoptosis; Angiogenesis

Core tip: Treatment of locally advanced rectal cancer stage Ⅱ and Ⅲ includes neoadjuvant chemo-radiation followed by surgery if clinically feasible. A strategy of observing patients without an operation has been proposed by some surgeons, but this is still the center of much debate. Moreover, the therapeutic effect of ionizing radiation in treatment of rectal cancer varies significantly from one person to another. This has led investigators to identify the molecular targets and pathways in rectal tumors resistant to ionizing radiation in a bid to improve the therapeutic effect of radiation by advanced biomedical and genetic engineering.

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INTRODUCTION

There are approximately 40340 patients diagnosed with rectal cancer annually in United States[1]. Cancer of the colon and rectum combined claimed 51690 deaths in 2012[1]. Rectal cancer, though staged similarly to colon cancer, is managed differently due to the pelvic location of the rectum. The rectum is in close proximity to the urogenital organs and anal sphincters. Hence, surgery for rectal cancer is associated with complications ranging from 15% to 70%[2]. Moreover, many patients will have local as well as distant metastasis during post-op surveillance[3,4]. Hence, careful and methodical planning

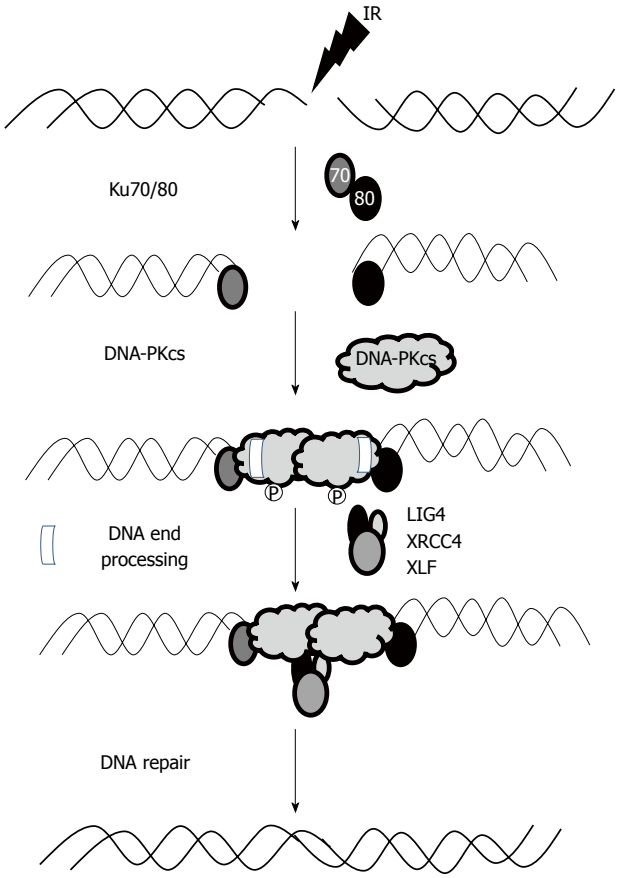

Figure 1 Schematic representations of double-strand break repair by non-homologous end-joining mechanism. The KU proteins are the initial participants in this process as they rapidly bind to broken DNA segments. Another major function of the KU proteins is the active recruitment of DNA-PKcs. DNA-PK activation assists with the recruitment of other proteins involved in the limited DNAend-processing (Artemis, pol m, pol l, and TDK) required to generate ligatable DNA ends. Ligation is mediated by the LIG4/XRCC4 complex and is assisted by the ligation mediator XLF. Once this process is completed, DNA integrity is maintained.

is required to avoid unnecessary surgery with potential short and long term complications. Recent studies have underscored the importance of ionizing radiation (as neoadjuvant therapy) in patients with stage Ⅱ and Ⅲ rectal cancer. There are many benefits to the use of IR in the neoadjuvant compared to the adjuvant setting[5]. Additionally, in some cases, this approach allows the tumors to be down-staged resulting in complete pathological response (pCR, *i.e.*, complete obliteration of the tumor following preoperative chemoradiation at laparotomy) or complete clinical response (cCR, *i.e.*, complete obliteration of the tumor following preoperative chemoradiation during repeat colonoscopy or other diagnostic modalities such as MRI).

However, the benefit from preoperative radiation varies significantly in trials with a substantially wide pCR (9%-37%)[6-10]. Patients who achieve a pCR have better outcomes compared to patients who do not[11]. Some surgeons have elected a watchful waiting approach for patients who achieve cCR[12-17].

The logical clinical and pre-clinical question is to devise methods by which we can personalize treatment for rectal cancer, such that the most effective therapy with the least side effect profile can be offered consistently to patients affected by rectal cancer. In order to achieve this objective, extensive research has been performed over the last few decades to identify biological markers and genetic phenotypes that can predict successful response to radiation and translate into improved survival. We present a review of the current status of these markers.

THE THERAPEUTIC EFFECTS OF IR

The NHEJ pathway of DNA repair

The therapeutic effect of IR is largely the result of double stranded DNA breaks that result from IR-induced DNA damage. DNA breaks are difficult to repair and typically result in apoptosis. DNA double-strand break (DSB) can be repaired by one of the following three pathways: homologous recombination[18], non-homologous end-joining (NHEJ) pathway, or an alternate NHEJ pathway (characterized by larger deletions and translocations)[19]. The details behind the selection and execution of these pathways are not entirely clear, but it seems that NHEJ is the major pathway as it is the only one that occurs in all stages of cell cycle.

The NHEJ pathway is essential for DSB repair and is also important for V (D) J recombination during T and B cell lymphocyte development. The catalytic subunit of DNA-dependent protein kinase (DNA-PKcs) is an integral part of the NHEJ pathway. The actual mechanism of this pathway is rather complex (Figure 1), but can be broadly classified into three steps. In the first phase, Ku 70/80 heterodimer identifies DSB, facilitates the activation and recruitment of DNA-PKcs, and then ties the DNA ends in a synaptic complex[20]. The next step involves enzymatic processing of the DNA ends followed by ligation (by DNA ligase Ⅳ) in the last phase. The order and timing of this sequence of events is not well defined; however, it is widely regarded that Ku 70/80 protein is the most important and integral part of this sequence as it recruits DNA-PKcs as well as interacts with a host of other important proteins. Moreover, Ku has lyase activity allowing it to process DNA ends during NHEJ[21].

Following successful DNA repair, the cell might undergo back to the normal cell cycle. If some error occurs during the repair, the cell might undergo genomic instability and if the cell is unable to repair the radiation-induced damage, it undergoes apoptosis (Figure 2)[22]. Thus, a logical place to begin investigating marker of radioresistance is by interrogating the NHEJ pathway of DNA repair in cancer cells.

Role of DNA-PKcs

DNA-PKcs has multiple roles in DNA repair and carcinogenesis. DNA-PKcs facilitates DSB repair, thus ensuring stability and integrity of genetic chromosomes. Hence, low levels of DNA-PKcs might result in muta-

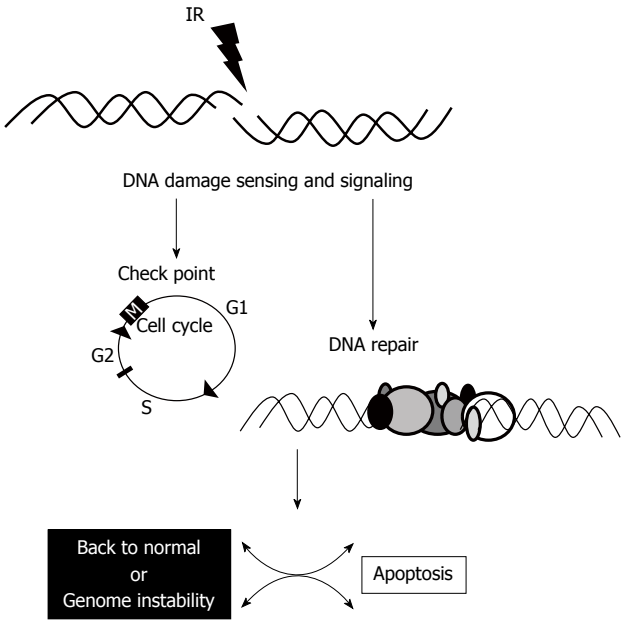

Figure 2 Schematic representation of the events that occur following IR-induced DNA damage. Sensing mechanisms and signaling first stop the cell cycle that allows the cell to repair the DNA damage. If unsuccessful, apoptosis ensues. If the repair is nearly complete, the cell might continue to replicate with genome instability.

tions promulgating the cascade of carcinogenesis. A cell with low levels of DNA-PKcs might be unable to repair the DNA damage incurred by IR and destine the cell for apoptosis. In this scenario, low levels of DNA-PKcs should be a surrogate for radiosensitivity.

On the other hand, cancer cells might contain higher DNA-PKcs levels induced by the rapid cell turnover. In this scenario, increases in DNA-PKcs activity will enhance cancer cell resistance and decrease susceptibility to chemotherapy and ionizing radiation[23-26].

Pre-clinical studies have demonstrated that DNA-PKcs deficient Chinese hamster ovary cells showed profound cell death following treatment with IR compared to the DNA-PKcs complimented V3-YAC cells[27]. Colon cancer HCT-116 DNA-PKcs$^{-/-}$ cells and xenografts were exquisitely sensitive to IR[28,29]. Unfortunately, the role of DNA-PKcs activity in development of various cancers has been investigated in multiple studies and has shown conflicting results in carcinogenesis as well as being a poor predictor of a response to IR, but more data is needed in this area (Table 1).

Significant increases of DNA-PKcs activity have been observed in certain gastrointestinal cancers such as colorectal cancer[30,31], esophageal cancer[32], nasopharyngeal cancer, and non-small cell lung cancer[33]. Conversely, loss of DNA-PKcs expression has been linked to gastric tumors correlating with signs of invasion and poor survival[34,35].

Levels of DNA-PKcs in cancer cells before treatment (radiation or chemotherapy) has been compared to levels after treatment, and have shown mixed results. The expression of DNA-PKcs was noted to be directly proportional to a favorable response with radiation in esophageal and early breast cancer but not in nasopharyngeal cancer[36-38]. On the other hand, studies have revealed increased levels of DNA-PKcs and Ku proteins in residual tumors after radiation treatment, suggesting a means of survival and a marker of radioresistance in recurrent tumors[39].

While the cellular status of the DNA-PKcs as a predictor of IR remains to be investigated, DNA-PKcs inhibition might have a therapeutic role in rectal cancer. Pre-clinical studies showed that pharmacological inhibition of DNA-PKcs led to substantial chemo- and radiosensitization[27,40-42]. The effect of DNA- PKcs inhibitors has been examined in mouse xenograft tumor models with favorable results. There has been significant tumor growth delay and improved survival in mice treated with combined DNA-PKcs inhibition and ionizing radiation. The combination treatment reduces levels of cell proliferation marker Ki67 and increases activity of certain proteins known for its anti-tumor properties[43,44].

Inhibitors of DNA-PKcs have been shown to have a synergistic effect along with cisplatinum/platinum based drugs in treatment of ovarian, colon, and breast cancer[45-47]. Multiple DNA-PKcs kinase activity inhibitors are not only in various stages of development but a few are being tested in clinical trials (Table 2). Similarly, new *in vivo* substrates of DNA-dependent protein kinase (Akt1/PKBa, Hsp90a, NR4A[48-52]), which can be induced by ionizing radiation have been identified.

Furthermore, additional DNA-PKcs inhibitors have been developed such as anti-DNA-PKcs ScFv 18-2 (derived from an existing anti-DNA PKcs monoclonal antibody)[53], and anti-DPK3-scFv (selected from a humanized semi-synthetic scFV library)[44]. These anti-DNA PKcs sensitize cells to radiation induced injury[44,54,55] in a similar fashion to RNA inhibition of DNA-PKcs transcripts[56-58].

The interaction between epidermal growth factor receptor (EGFR) and the DNA-PKcs has also been explored. This interaction is required for radiation induced nuclear AKT phosphorylation and cell survival[52,59,60]. Similarly, blockage of EGFR signaling pathway with a monoclonal antibody can inhibit DNA-PKcs activation and thereby decrease DNA repair capacity. This could enhance sensitization and susceptibility of cells to ionizing radiation[61,62].

Clinically, deficiency in DNA-PK activity led to sensitivity to nitrogen mustards in patients with chronic lymphocytic leukemia[25]. The drug 2-N-morpholino-8-dibenzothiophenyl-chromen-4-one (NU7441) is a potent and specific DNA-PK inhibitor[63]. Treatment with NU7441 and topoisomerase inhibitors combined with IR caused potent chemo-radio sensitization in SW620 colorectal cancer cells as well as xenografts[27]. The various mechanisms by which DNA-PKcs inhibitors facilitate radiation induced death include apoptosis[64,65], acceleration of senescence, induction of mitotic catastrophe, and autophagy[43,66,67].

Studies evaluating expression of DNA-PKcs in pe-

Table 1 Association between DNA-PKcs activity and cancer development from clinical investigations

Tumor type	Assay	Specimen	Sample size	DNA-PKcs activity	Interpretation
Nasopharyngeal cancer	IHC	Tumor	66	↑ in 70% of tumor tissue	No association with locoregional control and survival
Nasopharyngeal cancer	IHC	Tumor	223	↑ in 37% of tumor tissue	Overexpression associated with advanced stage and poor survival
Esophageal cancer	IHC, IB, Kinase activity	Tumor, normal	13 paired	↑ in tumor tissue	NA
Gastric cancer	IHC	Tumor	279	↑ in 73% of tumor tissue	Loss of expression associated with lymphatic invasion, lymph node metastasis, advanced pathological stage, and poor survival
Gastric cancer	IHC	Tumor, normal	791	↑ in 80% of tumor tissue	Loss of expression associated with intratumoral neutrophils, microsatellite instability, mutations in DNA-PKcs and poor survival
Colorectal cancer	RT-PCR, IB, kinase activity	Tumor, normal	12 paired	↑ in tumor tissue	NA
Colorectal cancer	IHC, IB	Tumor, normal	359 (35 paired)	↑ in 64% of tumor tissue	Overexpression associated with clinical stage, lymphatic invasion, distant metastasis and poor survival
Non-small cell lung cancer	IHC	Tumor	113	↑ in 89% of tumor tissue	Overexpression associated with tumor grade
Non-small cell lung cancer	IHC	Tumor	86	↑ in 87% of tumor tissue	No association with clinical characteristics or outcome
Non-small cell lung cancer	RT-PCR	Tumor, normal	140 paired	↑ in tumor tissue	Overexpression associated with poor survival
Non-small cell lung cancer	IHC	Tumor, normal	116 (12 paired)	↑ in 75% of tumor tissue	No association with clinical characteristics or outcome
Glioma	Kinase activity	Tumor	36	↑ in tumor tissue	Hyperactivity correlates with rumor grading
Ovarian cancer	IHC	Tumor, normal	100	↓ in 40% of tumor tissue	loss of expression associated with tumor progression, advanced clincal stage, and lymph node metastasis
ALL, CLL, lymphoma, multiple myeloma	IHC, IB	Lymphoid tissue	86	↑ During lymphoid development and in lymphoid malignancies	Overexpression associated with higher lymphoma grading and degree of maturation in lymphoid malignancies other than multiple myeloma
B-cell CLL	IB, kinase activity	Lukemia cells	54	↑ in del(17p) and del(11q)	Overexpression associated with shorter treatment free interval
B-cell CLL	RT-PCR	Lukemia cells	50	↑ in del(17p)	Overexpression associated with poor survival
Cancer of breast, cervix, head and neck esophageal and lymphoma	Kinase activity	PBLs	167	↓ in advanced stage	Hypoactivity associated with advanced stage and distant metastasis
Radiation response					
Esophageal cancer	IHC	Tumor	67	↑ in 54% of tumor tissue	Overexpression predicts better response to chemoradiation
Oral squamous cell carcinoma	IHC	Tumor	42	↑ in residual tumor after RT	Not predictive of radiation response
Cervical cancer	IHC	Tumor	22	↑ in residual tumor after RT	No association with clinical characteristics
Breast cancer	IHC	Tumor	224	↑ in 43% of tumor tissue	Overexpression predicts better locoregional control of radiation alone versus chemotherapy alone in early stage
Cancer risk					
Lung cancer	Kinase activity	PBLs	Cancer 41/healthy 41	↓ in cancer patients	Hypoactivity associated with cancer of the lung
Breast, cervix, head and neck, esophagus and lymphoma	Kinase activity	PBLs	Cancer 93/healthy 41	↓ in cancer patients	Hypoactivity associated with chromosomal instability and cancer of breast and cervix

Adapted with permission[148]. ALL: Acute lymphocytic leukemia; CLL: Chronic lymphocytic leukemia; IHC: Immunohistochemistry; PBLs: Peripheral blood lymphocytes; RT-PCR: Reverse transcription polymerase chain reaction; ↑: Indicates increase activity; ↓: Indicates decrease activity.

ripheral blood lymphocytes (PBLs) as a marker of host immunity and cancer development have shown an additional role in cancer development as it relates to host immunity. Data from multiple studies demonstrated that cancer patients have a lower level of DNA-PKcs activity in PBLs[23,68,69], suggesting impaired ability to recognize cancer cells leading to a poor prognosis. Whether this is mediated by activation of natural killer (NK) cells or release of pro-inflammatory cytokines is not clearly understood[70]. Destruction of NK cells leading to increases

Table 2 Non-homologous end-joining inhibitors

Inhibitor	Mechanism/comments
A12B4C3	PNKP inhibitor, sensitizes cells to camptothecin
BTW3	A small peptide DNA-PK inhibitor, proposed to compete for DNA-PKcs autophosphorylation
KU0060648	DNA-PK and P13K inhibitor
NU7441/KU57788	DNA-PK inhibitor, competitive with ATP
ScFv 18-2	An antibody-derived DNA-PK inhibitor that can bind to an epitope unique to DNA-PKcs
ZSTK474	DNA-PK and P13K inhibitor, competitive with ATP; in phase 1 clinical trials (NCT01280487 and NCT01682473)
CC-115	Dual inhibitor of DNA-PKcs and mTOR, in phase 1 clinical trials
CC-122	DNA-PK inhibitor, in phase 1 clinical trials

Reprinted with permission from Elsevier[149]. P13K: Phosphatidyl inositol 3 kinase.

in spontaneous tumor development in mouse models[71] leans in favor to the former hypothesis. Moreover, an inverse association between DNA-PKcs activity in PBLs and stage of cancer was also observed in patients who were treated with radiotherapy for advanced cancer, displaying poorer prognosis and higher frequency of distant metastasis[68].

In addition to its role in NHEJ pathway, DNA-PKcs regulates the DNA damage repair mechanisms by a variety of mechanisms. These include DNA interstrand crosslink (ICL) repair[72,73], AKT activation, EGFR nuclear translocation, or activation/mobilization of chromatin remodeling factor structure-specific recognition protein 1 (SSRP1) from nucleolus[60,74,75]. Biomedical engineering aiming to mimic some of the activities of the DNA-PKcs has been instrumental in developing novel agents that might be useful for cancer therapeutics.

It is clear that the status of the DNA-PKcs plays a fundamental role in ionizing radiation-induced cell death. Many aspects of its role in cancer therapeutics are currently under investigation. In rectal cancer, the role of DNA-PKcs is still in its infancy. As markers of a response to ionizing radiation, the role of the DNA-PKcs is complicated by the fact that there is paucity of high quality data. In rectal cancer, our group demonstrated counter-intuitive results with regards to the role of DNA-PKcs in the response to IR (discussed below). In prostate cancer, nuclear positivity for DNA-PKcs was associated with chemical recurrence[76]. Further studies are required to shed more light into these issues.

The Ku proteins

Ku70 and Ku80 proteins are essential components of the NHEJ pathway. These proteins serve as a medium by which multiple other DNA-repair proteins can be attached to the pathway cascade[77]. Importantly, the Ku proteins have a high affinity for broken DNA strands and rapidly bind to them. This initial process also recruits DNA-PKcs for DNA repair, though the exact mechanism is still unknown[78,79]. Additionally, Ku proteins play a major role in recruitment of XRCC4[80,81], XLF[82], APLF (APTX and PNK-like factor)[83] to DSBs helping with the repair process and promoting NHEJ. Moreover, Ku has the ability to enzymatically process DNA ends during NHEJ using the 5'-deoxyribose-5-phosphate (5'-dRP)/AP lyase activity[21]. Ku also excises abasic sites near DSBs suggesting a potential role in repairing damage by IR[21].

Intuitively, tumors that express high levels of Ku proteins should be able to repair the damage induced by IR more efficiently and thus become more resistant to therapy. *In vitro* studies have failed to show an association between the Ku proteins and radiosensitivity[84]. *Ex vivo* studies have also interrogated the role of the Ku proteins as surrogates of a response to IR.

Lack of Ku70 immunoreactivity correlated with radiosensitivity in patients with carcinoma of the cervix. In these patients, survival was better in tumors that had lower nuclear expression of Ku70[84]. In squamous cell carcinoma of the head and neck, Ku80 over expression was an independent predictor of regional recurrence and mortality in patient treated with IR[85]. Similarly, in rectal cancer low levels of Ku70 and Ku80 were associated with pCR. Ku70 was associated with down-staging. Disease free survival was 42% in patients with high Ku70 expression compared to 78% in patients with low expression of the same protein. Similar results were observed for Ku80[86]. Elevated levels of Ku proteins occur in high grade lymphoid malignancies[87]. The Ku70/Ku80 heterodimer DNA end-binding activity was 2- to 3-fold higher in the resistant B-CLL cell subset compared with the sensitive B-CLL cell subset[88], highlighting a possible mechanism behind increased DNA-PKcs activity in resistant CLL cells. The authors showed that novel DNA-dependent protein kinase (DNA-PK) inhibitor, NU7026 (2-(morpholin-4-yl)-benzo[h]chomen-4-one), and the phosphatidylinositol 3 (PI-3) kinase inhibitor, wortmannin, restored sensitivity to DNA damage-induced apoptosis of otherwise resistant cells.

Ku proteins can be upregulated after radiation treatment[39,89]. In one such study, expression of DNA-PK complex proteins (including Ku 70 proteins) increased after radiation treatment in residual tumors, and the increased values correlated with the tumor radiation resistance[89]. Various mechanisms have been postulated behind the role of Ku proteins in radioresistance. A distinct cell-interdependent signal is conveyed through gap junctions during chemotherapy with cisplatin, mediated by

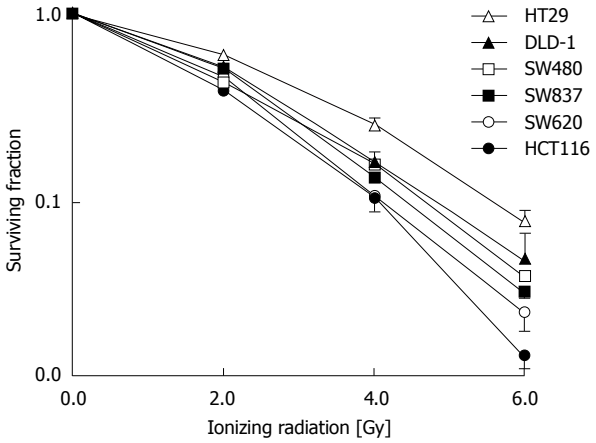

Figure 3 Response to ionizing radiation in several colorectal cancer cell lines subjected to various doses of ionizing radiation. There is a variable response to the same doses or ionizing radiation (Gy).

the kinase function of Ku70, Ku80 and DNA-dependent protein kinase complex. This communication may explain the resistance to cisplatin-induced death of cancer cells[90]. It is also possible that the role of Ku proteins and DNA-PKcs in DNA damage repair depends upon the extent and complexity of damage by IR. Studies have revealed that simple DSBs induced by laser irradiation are repaired rapidly involving Ku70/80 and XRCC4/Ligase IV/XLF. In contrast, DSBs with greater chemical complexity are repaired slowly and requires additional use of DNA-PKcs[91].

While these data seem compelling, more research is required prior to establishing the role of the Ku proteins in a response to radiation in rectal cancer. Current data on this subject, while promising, is currently limited and not clinically available. In rectal cancer, our group demonstrated counter-intuitive results with regards to the role of DNA-PKcs in the response to IR (discussed below).

ANALYSIS OF GENOTYPIC ORIGINS OF RADIORESISTANCE *IN VITRO* AND *IN VIVO* MODELS OF RECTAL CANCER

Examination of factors leading to radioresistance can practically be approached *in vitro*. Analysis of five colon cancer cell lines (HT29, DLD-1, SW480, SW620, and HCT116) as well as one rectal cancer cell line (SW837) have demonstrated a similar pattern of response to a group of patients treated for rectal cancer with pre-operative IR (Figure 3)[92]. The cell lines that have been treated with IR and examined originate from patients with different characteristics.

SW480 cells were derived from a primary Duke's stage B colon adenocarcinoma from a 50-year-old Caucasian male, while the SW620 cell line was cultured from a lymph node metastasis from the same patient at a later time. The DLD-1 cell line was established from an adult male with adenocarcinoma of the colon. The SW837 cell line was derived from a 53-year-old Caucasian male with rectal cancer. HCT-116 cells were cultured from an adult male with colon cancer. HT-29 cells were derived from a 44-year-old Caucasian woman with colorectal adenocarcinoma. All of these cells have mutations of the p53 gene, except for HCT-116 cells (p53-Wt). HT-29 cell have mutations of both alleles of the p53 gene (p53-null)[92]. HCT-116 cells display microsatellite instability.

These cells have been extensively studied and a number of properties are known. Analysis of these factors and a response to IR has not yielded any uniform patter of predictability that could be surrogate markers in *ex vivo* studies. For instance, the inhibitor of apoptosis, survivin, has been shown to play a significant role in resistance to IR (discussed below)[93]. Analysis of this model of rectal cancer *in vitro* (Figure 3) has not consistently corroborated this finding. For instance, survivin was expressed in higher levels in the radiosensitive SW620 compared to the relative more radioresistant SW480 cell line. Interestingly, these two cells originated from the same patient one at the time of stage Ⅱ colon cancer (SW480) and the second one from a lymph node metastasis (SW620) such that these two cell lines contain similar genetic background.

Analysis of these cell lines is representative of the response that was observed in 117 patients who were treated with preoperative ionizing radiation and underwent surgical resection (Figure 4). A pivotal question is to determine what causes these differences in patients and cell lines receiving the same treatment. A simple approach in the laboratory is to take the more radioresistant and the more radiosensitive cells and analyze specific differences. This approach has been undertaken *in vitro* and *in vivo*. HCT-116 cell and xenografts are substantially more sensitive to IR compared to HT-29 cells and xenografts (Figure 5).

DNA repair in this model

Analysis of DNA induced damage (by γH2AX) indicated that the radioresistant HCT-116 cells suffer more DNA damage when exposed to IR and that this damage persists over time indicating a poor ability of the cells to repair the DNA affected by IR (Figure 6)[94]. Predictably, HT-29 cells should be able to repair DNA more effectively and should have increased levels of DNA-PKcs and Ku proteins. In fact, the opposite results have been observed in our studies. Our results showed that compared to HCT-116 cells, HT-29 cells expressed lower levels of DNA-PKcs and Ku proteins[95].

Cell cycle kinetics in this model

Examination of cell cycle kinetics demonstrates that the radiosensitive HCT-116 cells substantially accumulate in the G-2 phase of the cell cycle. HT-29 cells proceed through the cell cycle in spite of receiving the same dose of IR (Figure 7)[22,28,92,94,96,97]. According to these observations, there should be differences in cell cycle regulators and apoptotic factors that could be used to predict a response to IR.

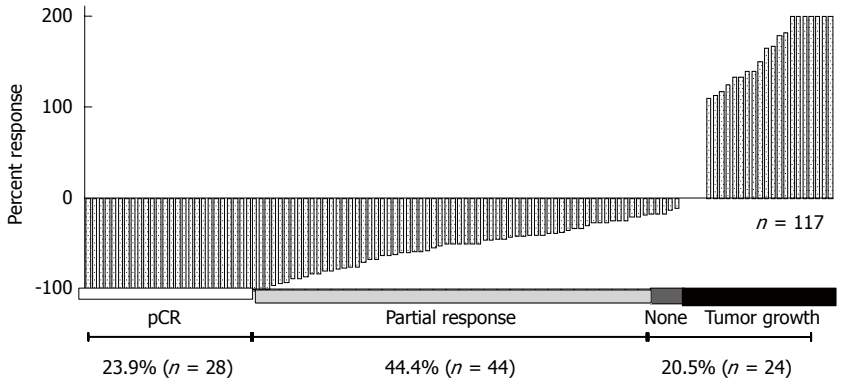

Figure 4 There is a high variability of a response to ionizing radiation in rectal cancer patients treated with pre-operative ionizing radiation. Each bar on the X-axis represents an individual patient. The Y-axis represents the clinical response to pre-operative ionizing radiation. Nearly one fourth of patients achieve a pCR, but close to another fourth do not respond to the same form of treatment, while the rest of patients have achieve a partial response.

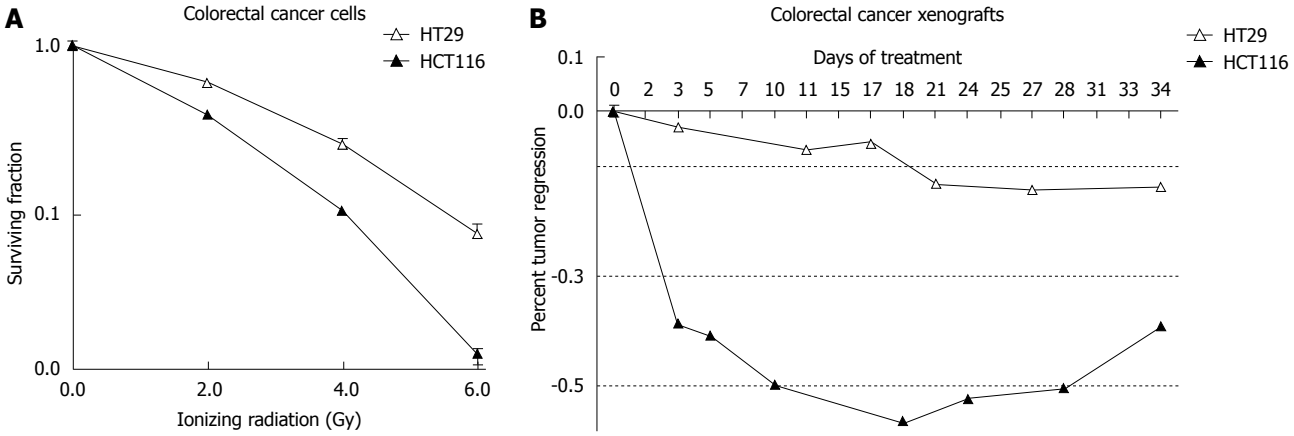

Figure 5 Analysis of the most radiosensitive (HCT116) and the most radioresistant (HT29) cells (A), a similar response has been noted in cells implanted in immune compromised mice bearing xenografts of these cells (B).

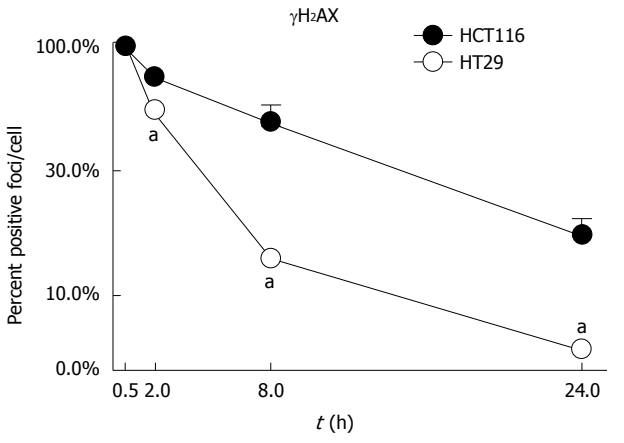

Figure 6 Analysis of DNA-induced damaged by ionizing radiation as determined by γH2AX. HCT116 cells undergo more pronounced damage when treated with the same dose of ionizing radiation compared to HT29 cells. The damage induced in HCT116 cells persists over time. [a]$P < 0.05$ *vs* HCT116.

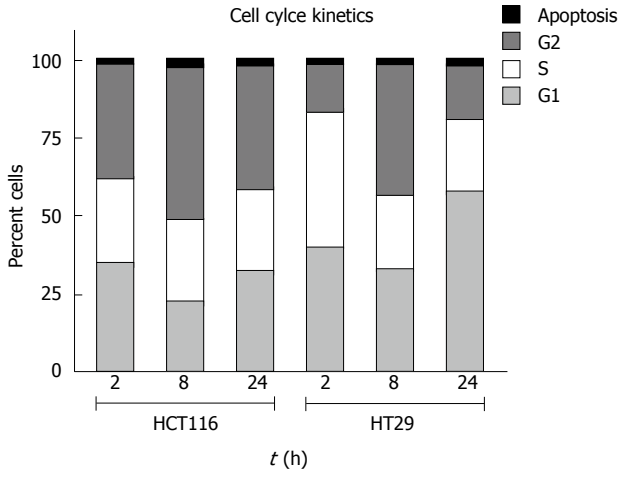

Figure 7 Cell cycle kinetics of HCT116 and HT29 cells treated with 2.0 Gy ionizing radiation. There is a pronounced accumulation of cells in G2 in HCT116 cells. HT29 cells continue through the cell cycle in spite of receiving the same dose of ionizing radiation.

Apoptosis in this model

Analysis of this model with regards to the central mediators of apoptosis (as depicted in Figure 8) has demonstrated the following in HCT116 (*vs* HT29 cells): marked over expression of p21, decreased expression of p53, Bax, Bcl-2 and survivin[92]. Examination of these findings is intuitive in some areas while counterintuitive in oth-

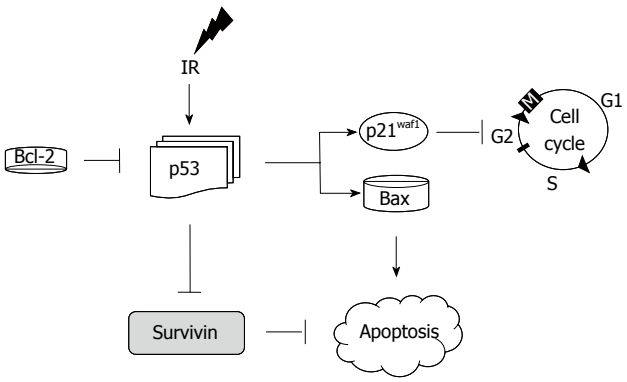

Figure 8 Schematic representation of molecular events following the cellular response to ionizing radiation-induced damage. Ionizing radiation causes an up-regulation of p53. p53 then directly activates the cyclin dependent kinase inhibitor p21. Cell cycle progression stops until the cell repairs the damaged induced by ionizing radiation. If the cell is unable to repair itself, it undergoes apoptosis. Bcl-2 inhibits p53 up-regulation, while p53 inhibits the inhibitor of apoptosis: survivin.

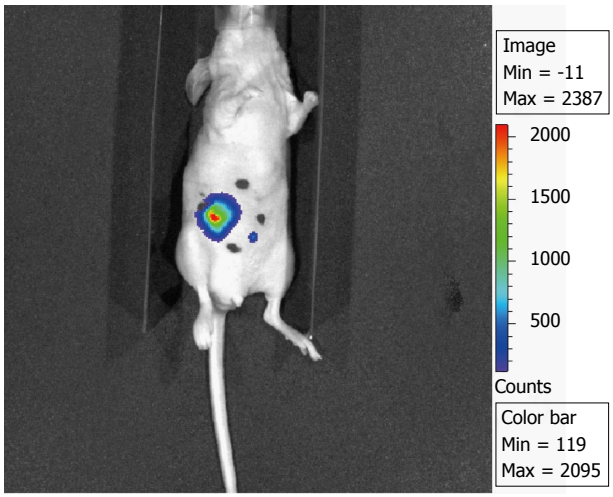

Figure 9 Orthotopic model for the study of rectal cancer. This model has the following characteristics: (1) cecal transplantation of tumors with a known response to ionizing radiation; (2) attachment of the cecum to the lateral abdominal wall with a permanent suture for the administration of ionizing radiation; and (3) transfection of cells with luciferase before tumor implantation for the assessment of the chemoradiotherapeutic interventions over time by bioluminescence imaging before the end of the study. This technique allows targeted delivery of ionizing radiation in an intraperitoneal tumor.

ers. For instance, p21 elevation in response to IR is an expected response of these radiosensitive cells. This was associated with an appropriate response of p53 leading to activation of p21 culminating in apoptosis as demonstrated by an elevation of the cleaved PARP-1. In HT29 cells, on the other hand, p53 was markedly elevated. This is the result of the mutated status of p53 in HT29 cells. However, the results with regards to Bax and survivin are not clear in these experiments as a decrease in survivin and Bax was expected in these radioresistant cells.

In separate *in vitro* studies, analysis with colorectal cancer cells with stable knock out (KO) of genes responsible for apoptosis from IR-induced injury was undertaken. This demonstrated that the p21 and the Bax KO genotypes were associated with radiosensitivity rather than radioresistance (Figure 6)[28]. The results with regards to p21 have been previously reported and indicate that it is mitotic catastrophe that leads these cells to undergo cellular death rather than becoming more radioresistant. The Bax KO genotype leading to a more radiosensitive phenotype as opposed to radioresistance was partly mediated by apoptosis inducing factor (AIF) and not to caspase mediated apoptosis[28]. AIF is an important mediator of cellular death that requires further studies as a predictor of a response to IR in rectal cancer[98].

These observations *in vitro* have been noted *in vivo* models of rectal cancer as well. However, one of the limitations of the studies *in vivo* is that these studies have relied on xenograft models of rectal cancer. We have previously described an orthotropic model in which cells have been implanted in the cecum and then the cecum was secured to the abdominal wall for targeted IR. Because these cells can be labeled with luciferase, the response to IR can be followed over time by biluminescence imaging (Figure 9). However, this model requires further validation[97].

In summary, observations from these studies demonstrate that there are good models for the study of rectal cancer in response to IR *in vitro* and *in vivo*. We have identified some molecules that can be used to predict a response to IR in HT-29 and HCT-116 cells. Application of these factors to the rest of the cells as depicted in Figure 3 has yielded mixed results. There is no unifying pathway that has been identified to date. Moreover, identification of predictors for a response to IR remain at large. For instance, many inhibitors of apoptosis examined (IAPs; survivin, XIAP, cIAP 1/2) were all increased in the more radiosensitive SW620 cells compared to the SW480 cells. Survivin, in response IR in colorectal cancer cells (0, 2, 4, and 6 Gy) was expressed in the following order in several cells: SW620 > HT-29 > HCT-116. Apoptosis was interrogated by PARP-1 cleavage and demonstrated that apoptosis in response to IR occurred in the following pattern: DLD-1 > HCT-116 > SW480 > HT-29 > SW480. p27 demonstrated the following pattern: HT-29 > HCT-116 > SW480. There was no particular pattern of expression of these factors nor was there a correlation to a response to IR noted. Thus, there is further need for identification of a unifying pathway that could be used to determine a response to IR.

The additional advantage of the current *in vitro* and *in vivo* models is that they can be utilized for the study of radiosensitizing agents and some of these have demonstrated promising results[92,94]. The effects of the radiosensitizing agents on specific pathways can also be explored in this fashion.

We then proceeded with a review of literature to determine how these observations compared to other studies. The result of this review have been previously documented to some extent and are presented and updated in the following discussion[22,99].

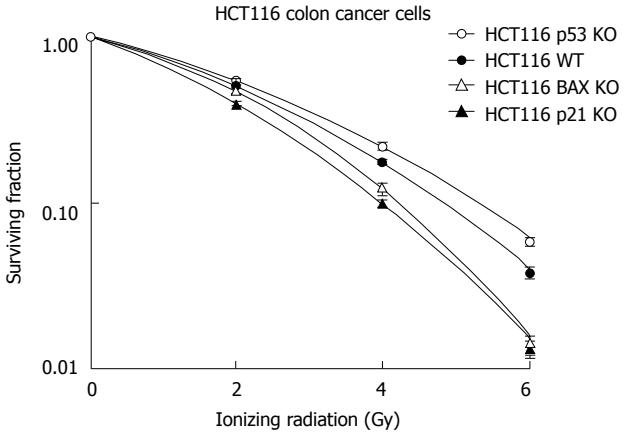

Figure 10 Analysis of HCT116 cells with stable KO genotypes for p53, p21, and Bax compared to wild-type. Cells deficient in p53 are more radioresistant, while p21 and Bax deficient cells are more radiosensitive compared to wild-type.

FACTORS THAT LEAD TO A RESPONSE TO IR: A REVIEW OF THE LITERATURE

Apoptosis

If cells are unable to repair the damage induced by IR, the cell is destined to undergo programmed cell death. In the classical pathway, the stressed cell leads to an up-regulation of p53, which then stops the cell cycle *via* induction of the cyclin depended kinase inhibitor p21. Failure to repair the damage causes BAX to induce apoptosis[22,100] (Figure 8).

It is conceivable that defects in any of these molecules (apoptotic or cell cycle proteins) alone or in combination could serve as a surrogate to predict a response to IR in rectal cancer. *In vitro* studies with colon cancer cells exposed to radiation have been in agreement with the classical response to apoptosis with p53, but not uniformly with p21 and BAX (as discussed in the previous section)[28] (Figure 8).

Apoptotic proteins: p53, p21, BAX, Bcl-2, survivin, and SMAC/Diablo

p53: *In vitro*, HCT-116 cells deficient of p53 are more radioresistant compared to HCT-116 wild-type cells. Tumor xenografts derived from the same cells demonstrated a similar effect[28]. These results have been mirrored in models of colorectal cancer *in vitro* and *in vivo*[101,102], but in disagreement with others[103-105]. Other studies have suggested that p53 mutations may render cells more radiosensitive owing to a reduction in p53-dependent DNA repair mechanisms[106]. Thus, *in vitro* and *in vivo* studies with regards to p53 have shown mixed results. *In vitro*, data indicates that lack of p53 leads to radioresistance. However, the mutational status of p53 is important to consider in all analyses examining p53[22].

Ex vivo studies have demonstrated a number of heterogeneous findings as well. Some studies have shown that mutated p53 leads to radioresistance in rectal cancer tissues[107]. Nuclear expression of p53 in rectal cancers predicted treatment failure and signified resistance to preoperative IR[96]. Other studies have demonstrated no usefulness of p53 as a marker of a response to IR[108,109]. To date, *ex vivo* studies have failed to provide usefulness as a marker of a response to IR. This might be the result of the low number of subjects included in the studies, the wide range of techniques utilized to detect p53, or the ability of the antibody to recognize the mutated *vs* the wild-type form of p53[22].

Cell cycle factors such as p53 and the cyclin dependent kinase inhibitors (CDKIs) (p21 and p27) have been studied as possible candidates to predict a response to ionizing radiation in rectal cancer. p21 is the classical CDKI and is activated by p53[110,111]. Irradiated colon cancer DLD-1 cells expressed low levels of p21[112]. The expected response to IR in cells and tumors deficient of p21 would be a radioresistant phenotype. Recent studies have shown that HCT-116 cell deficient of p21 are, in fact, more sensitive to ionizing radiation compared to wild-type HCT-116 cells[28,113]. Tumor xenografts deficient of p21 demonstrated more tumor regression compared to the wild-type genotype treated with the same dose of ionizing radiation[28].

p21: *Ex vivo* studies demonstrated the p21 positive tumors had a good response to IR[114]. Another study showed that p21 expression correlated with good pathological response and tumor radiosensitivity[115]. Similarly, a reduction by 50% in post-irradiated rectal tissue compared to pre-irradiated one was associated with radioresistance[116]. Another study did not find p21 useful as a predictor of a response to IR[117].

p27: This study found that p27 positive tumors had a better response to IR with an OR of 3.3[117]. Similarly, the absence of p53 and p27 prior to treatment was associated with poor response to IR in rectal tumors[118].

Bax: Bax is a pro-apoptotic protein that leads to the release of cytochrome c from the intermitochondrial membrane[100]. It may be anticipated that Bax deficiency would be associated with radioresistance. *In vitro* and *in vivo* studies have demonstrated the opposite phenotype to IR (Figure 10)[28]. While a few studies demonstrate that Bax deficient cells are resistant to chemotherapeutic agents[119-121], evidence indicating the response of Bax deficient colorectal cancer cells to IR in pre-clinical studies is lacking. Limited *ex vivo* studies have shown that Bax tumor expression had a positive response to chemoradiation in patients treated for rectal cancer[122,123].

Bcl-2 inhibits cellular apoptosis and is overexpressed in many colorectal tumors[124]. BAX is the apoptogenic counter part of Bcl-2. Current studies have failed to demonstrate the association of Bcl-2 as a marker of response to IR[22,123,125].

Survivin: Survivin is one of eight inhibitors of apopto-

sis (IAPs) that are generated *via* induction of NFκB[100]. Survivin binds and inactivates caspases 3, 7 and 9[100]. *In vitro* and *in vivo* data showed that the NFκB-IAPs axis is a predictor of a poor response to IR when over expressed[22]. *Ex vivo* data supports the role of survivin in raidoresistance[93]. Furthermore, the five year survival of patients with survivin positive stage Ⅱ colon cancer tumors was 41% lower than patients with survivin negative tumors[126]. The role of other r IAPs (*i.e.*, XIAP, cIAP, *etc.*) and a response to IR remains at large.

The role of the IAPs in response to IR has been further interrogated by directly inhibiting the inhibition of the IAPs *via* augmentation of an antagonistic factor to the IAPs: SMAC/Diablo.

SMAC/Diablo: Pro-apoptotic molecules with the ability to reduce the functional activity of the inhibitors of apoptosis might have potential therapeutic applications. Compounds that mimic the action of SMAC/Diablo (Smac-mimetics) are under study for their ability to chemo- and radiosensitize tumor cells[127]. The Smac mimetic JP-1201 radiosensitized HT-29 colorectal cancer cells and xenografts by a marked augmentation in apoptosis, which was associated with a reduction in the levels of the IAP XIAP[94].

Proliferation markers and mitotic index as markers: A few studies have reported high Ki-67 staining correlated with a positive response to IR[128,129]. In contrast, most studies have demonstrated that proliferating nuclear antigen labeling index does not correlate with response to IR[115,125,130].

Apoptotic index: Evaluation of apoptosis in cancer cells has shown that patients with higher pre-radiation level of apoptosis (apoptotic index) had lower rate of recurrence and longer disease free period after radiation[131].

Logically, tumors that have an intact machinery to undergo apoptosis should respond better to ionizing radiation rather that those with mutation of one or more pro-apoptotic factors or activation of anti-apoptotic factors. Caspase mediated apoptosis has been shown to play a promising role in predicting a response to IR. A high spontaneous apoptotic index in pretreated tumor tissue was associated with a superior rate of response to radiation[132]. Furthermore, in a large study including 465 pre-irradiated biopsies tumors underwent immunohistochemistry staining against the active form of caspase 3. This study showed that tumors with a high apoptotic index had less recurrence and a higher disease free survival[131].

While these results seem promising, uniformity across studies has not been established nor substantial reproducibility or adoption to clinical practice. The practical usefulness of this approach is limited by the dynamic process of apoptosis and by the wide variety of measurements and laboratory standardizations. The individual evaluation of specific molecules in the process of apoptosis either as a single factor or in combination with others seems to suffer from the same issues.

Hypoxia and angiogenic factors

Hypoxia: Lack of oxygen supply to cancer cells has been linked to poor response to radiation. This premise was tested in patients undergoing neoadjuvant therapy for rectal cancer with the assistance of positron emission tomography using the copper-60-diacetyl-bis (N4-methylthiosemicarbazone (^{60}Cu-ATSM), an agent that accumulates in tissues lacking adequate oxygenation. Tumors with higher baseline tumor-muscle activity ratios (suggesting hypoxia) in the pre-treatment PET scan were shown to have a poor response to radiation[133]. Other agents tested in different studies have been less useful probably as a result of technical limitations[134].

Further evidence of the role of hypoxia in response to IR was demonstrated by the fact that higher levels of HIF-1 (hypoxia inducible protein factor 1, a protein that increases in oxygen deprived tissues) predicts poor response to neoadjuvant chemotherapy in patients with rectal cancer[135]. Additionally, HIF-1 correlates with increased levels of pro-angiogenic vascular endothelial growth factor (VEGF), a marker of angiogenesis for tumor growth[136].

VEGF: Low levels of VEGF have been associated with improved response to radiation[135,137,138], and vice versa[135,137-139]. Therefore, VEGF inhibition with the antibody bevacizumab has shown beneficial effects in treating cancers with neoadjuvant chemoradiotherapy[137,140,141]. Various mechanisms by which VEGF inhibition causes this effect may include reducing vascular density within a tumor, decreasing interstitial tumor pressures, improving global oxygenation status, vascular normalization and thus increasing responsiveness of endothelial cells to radiation[137,141,142]. It seems logical that if bevacizumab were to be used as a neoadjuvant agent in combination with IR for the treatment of patients with rectal cancer, these should have a higher rate of pCR compared to standard treatments. However, this observation has not been validated in clinical trials[143].

EGFR signaling: Initial reports revealed that combination of radiation and EGFR inhibition exerted a synergistic cytotoxic effect and hence raised interest in developing EGFR inhibitors. Hence, multiple EGFR inhibitors (*e.g.*, cetuximab and panitumumab) were developed and tested and have demonstrated promise in patients with KRAS wild-type tumors. However, with regards to the usefulness in EGFR signaling as a predictor of a response to IR, the data is lacking. Similarly, data pertaining to the usefulness of inhibiting the EGFR signaling pathway as a radiosensitizing modality has also demonstrated disappointing results[144].

High-throughput analyses

Microarray analysis: Single molecules as independent factors or in combination with other molecules of specific pathways (*i.e.*, apoptosis or angiogenesis) have not provided to be clinically useful to date. A major limita-

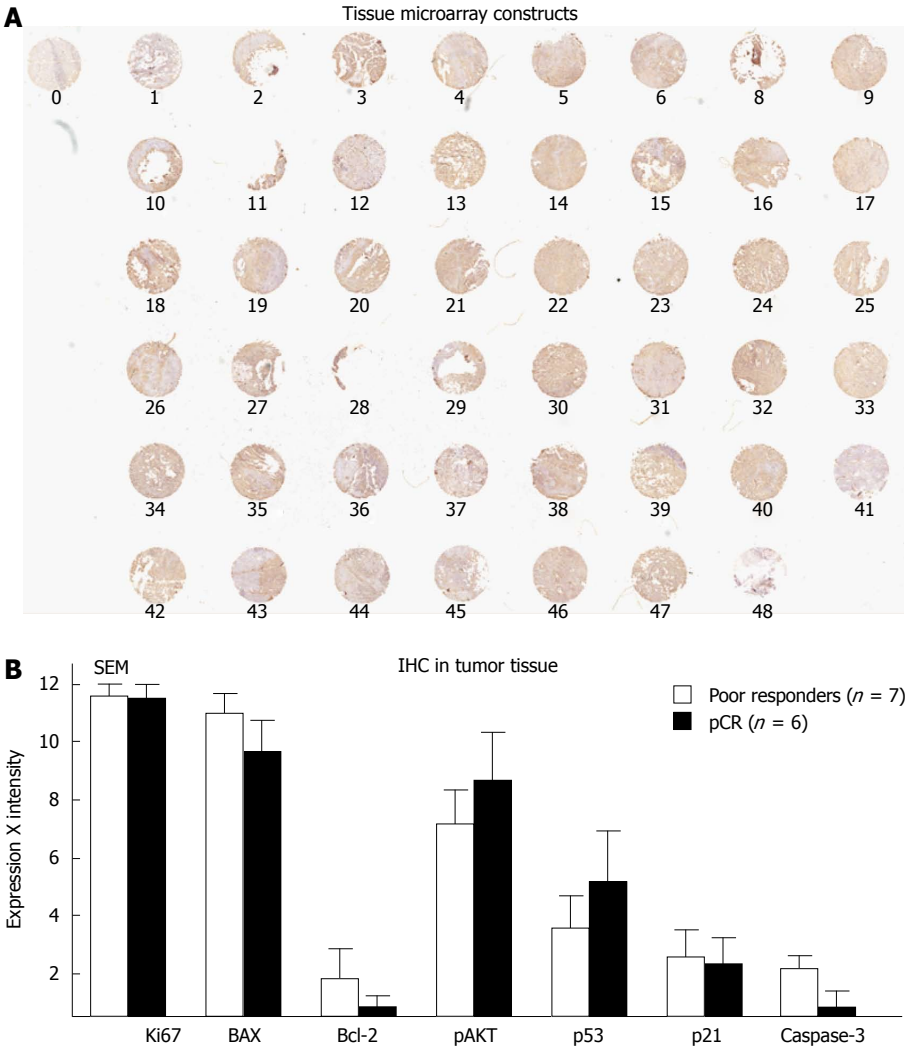

Figure 11 Tissue Microarray Constructs were created with 48 patients with rectal cancer that received pre-operative radiation. A: Of these 48 patients (each dot represents a patient), six had a pCR and seven did not respond to treatment; B: The differences in various tumor markers comparing these two groups. IHC: Immunohistochemistry; SEM: Scanning electron microscope.

tion of examining a specific pathway had to do with the dynamics of the process and the particular point in time at which it is being measured. Further, many tumors are heterogeneous in terms of mutations and alterations. Thus, interrogating several genes or proteins simultaneously is a logical approach in terms of elucidating origins of radioresistance in rectal cancer. In the era of personalized care, these tumor "fingerprints" not only make sense, but is the direction of the future.

Unfortunately, as appealing as it might seem, current efforts have been unsuccessful. Two studies have independently performed RNA arrays to analyze radioresistant and radiosensitive tumors. These studies have had limited genes and have had different results[145,146].

Tissue microarray: Tissue microarray is another technique to assess multiple proteins with a single experiment with tissues handled in a similar fashion. In one study, tissue microarray was performed with the goal of predicting survival and recurrence in patients treated with chemoradiation. In this study, Cox-2 emerged as a potential predictor of survival[147]. In a second study, our group subjected rectal cancer tissue to tissue microarray and tested eight different antibodies. MIB was the only independent predictor of a response to chemoradiation[8]. In our analysis, we examined tissue microarray in 48 patients who were treated with preoperative IR. We then divided all of these patients in two groups: patients who achieved a pCR (n = 6) compared to those who did not respond to IR or patients who experienced tumor growth (n = 7) in spite of pre-operative chemoradiation. We stained the tissue microarrays with seven antibodies and demonstrated no particular protein that could be used to differentiate these groups (Figure 11)[8].

CONCLUSION

Rectal cancer is the ideal clinical problem where personalized treatment could be investigated. This theory stems from the fact that a select patient population obtains an excellent response from the same form of chemoradiation, while others do not. Despite putting forward multiple mechanisms of tumor death from ionizing radiation and various possible causes of radioresistance, there

has not been a unifying pathway that can reliably predict a response to IR *in vitro*, *in vivo* or *ex vivo*. It is difficult to explain the reasons behind a clear discrepancy in the current observations in the literature. However, differences in tumor biology, genotypic profiling or phenotypic characteristics are some of these factors. There are currently good *in vitro*, *in vivo*, and *ex vivo* models for the study of rectal cancer and the trend seems optimistic in developing a predictive finger print for patients with rectal cancer that might respond well to IR. Recent data has shown that DNA-PKcs and Ku proteins (as vital players in NHEJ pathway allowing DSB repair) may have a central role in radiation induced cell death. Nevertheless many facets of its function in conjunction with the complex and intricate details of the pathway are still under investigation. More data is required before we can formulate one unified explanation for the heterogeneity noted in therapeutic effect of ionizing radiation. Until then, the hope of developing novel therapies for rectal cancer and improving the therapeutic yield of ionizing radiation with radiosensitizers remains a challenging clinical problem. The findings so far should not be viewed in a pessimistic fashion. There are several pathways that have provided potential targets for chemoradiotherapeutic interventions. We need to continue to investigate potential molecules predictive of a response to IR. As we dwell into the future, we need to remember that markers predictive of an aggressive behavior are currently in clinical practice such as testing for BRCA or RET proto-oncogene mutations. A view into the future also includes investigating base line characteristics of patient's genotypic background in normal tissue compared to tumor tissue after IR. It is important to determine if a patient starts with high levels at base line, but a particular gene is not activated then the base line levels are not as predictive. In the opposite scenario, we might have a patient with a molecule that at base line is low, but it is activated substantially with IR. In that scenario, we might consider those features as more predictive. The future, therefore, should be viewed with optimism.

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REVIEW

Advances and new perspectives in the treatment of metastatic colon cancer

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Abstract

During the last decade we have witnessed an unprecedented outburst of new treatment approaches for the management of metastatic colon cancer. Anti-angiogenic drugs, epidermal growth factor receptor blockers and multi-kinase inhibitors have all resulted in small but consistent improvement in clinical outcomes. However, this progress has paradoxically leaded us into new challenges. In many cases the clinical development was done in parallel and the lack of head-to-head comparison evolved into circumstances where several valid new "standards of care" are available. Even though desirable in essence, the availability of many options as well as different possible combinations frequently leaves the busy clinician in the difficult situation of having to choose between one or the other, sometimes without solid evidence to support each decision. In addition, progress never stops and new agents are continuously tested. For these reason this review will try to summarize all the clinical trials that constitute the theoretical framework that support our daily practice but will also procure the reader with rational answers to common clinical dilemmas by critically appraising the current literature. Lastly, we will provide with a compilation of promising new agents that may soon become our next line of defense against this deadly disease.

Key words: Colon Cancer; Stage Ⅳ; Metastatic; Review; Bevacizumab; Cetuximab; Panitumumab; Aflibercept; Regorafenib

Core tip: This manuscript is a comprehensive review, with the most updated information up to 2014, regarding metastatic colon cancer. It summarizes all those relevant clinical trials that constitute the theoretical framework to support our daily practice and provides rational answers to common clinical dilemmas. Additionally, it gives the reader a compilation of potential new agents that are currently being tested and may soon become the next step in the battle against this disease.

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INTRODUCTION

Colon cancer is the second leading cause of cancer-related mortality in the United States and 1.2 millions of

new cases are yearly diagnosed worldwide[1]. From the clinical perspective colon cancer could be categorized into the early stages (Ⅰ-Ⅲ) and the more advanced and usually lethal metastatic disease. Notably, since the publication of the MOSAIC trial almost ten years ago, no other groundbreaking development in the treatment of resectable colon cancer became available[2]. On the contrary, during the last decade we have witnessed an unprecedented outburst of new treatment approaches for the management of stage Ⅳ colon cancer that ultimately evolved into the approval of five new drugs. For simplification purposes, we can subdivide these new drugs into three categories: anti-angiogenic, epidermal growth factor receptor (EGFR) blockers and multi-kinase inhibitors. All of them represent important advances in the fight against this deadly disease. Nonetheless, some issues deserve further attention. First, these new agents were generally combined with at least some of the previously effective chemotherapy regimens (fluoropyrimidines and/or oxaliplatin and/or irinotecan). Also, the clinical development was done in parallel instead of following a rational stepwise approach where each new drug was tested against the new standard of care. This lack of head-to-head comparison resulted in several valid new "standards of care". Lastly, new combinations are continuously tested making extremely difficult for the busy clinician to keep up with the most updated information.

For the reasons mentioned before, this manuscript will pursue three clear objectives. First summarize all those relevant clinical trials that constitute the theoretical framework to support our daily practice. Second try to provide rational answers to common clinical dilemmas by critically appraising the current literature. Finally, provide the reader with a compilation of potential new agents that are currently being tested and may soon become the next step in the battle against this disease.

ANTI-ANGIOGENESIS AS A TARGET

Angiogenesis consists in a complex multistep process of new vessel formation. The vascular endothelial growth factor (VEGF) and its receptor (VEGFR) play a crucial role in the tumor transition from the "avascular" to the "vascular" phase, acquiring metastatic potential[3,4]. It also stimulates tumor growth, migration and metastasis through mechanisms not entirely related to tumor angiogenesis[5]. Moreover, tissue interstitial pressure is a key factor in chemotherapy delivery and in some tumors this could be up to 15 times higher than the normal counterparts[6]. There is solid evidence that VEGFR inhibition partially restores interstitial fluid pressure and reduces abnormal vasculature with improvement of drug delivery and enhancement of chemotherapy efficacy[7].

Bevacizumab

Bevacizumab (Avastin®, Genentech Inc.), a recombinant humanized monoclonal IgG-1 antibody against soluble VEGF-A, was the first anti-angiogenic drug approved for metastatic colon cancer. It prevents the binding of VEGF-A to the VEGFR and, consequently, inhibits angiogenesis, tumor growth and metastatic development. It was first approved on February 2004 by the FDA as first-line treatment for patients with metastatic colon cancer. Today, almost 10 years later, a substantial body of evidence has accumulated to help clinicians in the judicious use of this molecule. Table 1 summarizes the most relevant clinical trial of the anti-angiogenic drugs.

The first practice-changing, double blind, randomized phase Ⅲ trial that was published compared the use of irinotecan, bolus 5-FU and leucovorin (IFL) with or without bevacizumab in metastatic, previously untreated patients[8]. The primary endpoint of the study was overall survival (OS); disease-free survival (DFS) and overall response rate (ORR) were secondary endpoints. OS (20.3 mo *vs* 15.6 mo; $P < 0.001$) and PFS (10.6 mo *vs* 6.2 mo; $P < 0.001$) and ORR (45% *vs* 35%) were all significantly improved with bevacizumab. Importantly, patients in the IFL group were not allowed to crossover. Similar results were obtained in the ARTIST trial using a modified version of IFL (5-FU was infused over 6-8 h) plus bevacizumab in metastatic colon cancer, chemotherapy naïve, Chinese patients, confirming that results obtained in Caucasians were also applicable in Asian population[9]. Subsequently, in 2007 results from the BICC-C trial were released showing that bevacizumab combined with the classical bolus and 46-h infusional 5-FU plus leucovorin and irinotecan (FOLFIRI) was superior to a shorter version of IFL as upfront therapy[10]. In the original trial design patients were randomly assigned to receive FOLFIRI, IFL or irinotecan plus capecitabine (CapeIRI) with or without celecoxib. However, after the FDA-approval of bevacizumab the protocol was amended and additional 117 patients were randomized to receive bevacizumab with FOLFIRI (FOLFIRI-B) or IFL (IFL-B); due to excessive toxicity the CapeIRI arm was discontinued. With an updated median follow-up of 34.4 mo, OS was longer in the FOLFIRI-B arm (28.0 mo *vs* 19.2 mo; $P = 0.037$)[11]. Thus, infusional 5-FU regimens should be preferred over bolus 5-FU when combined with bevacizumab.

After the initial success with irinotecan combinations, bevacizumab was soon studied in oxaliplatin-based regimens. The first evidence of its synergistic effect came from the ECOG-3200 study that investigated the role of bevacizumab in the second line treatment[12]. In this study patients who had progressed to irinotecan and fluoropyrimidine therapies but who had not received oxaliplatin or bevacizumab were randomized to FOLFOX-4 (control arm), FOLFOX-4 plus bevacizumab (FOLFOX-B) or single agent bevacizumab. With a median follow-up of 28-mo, a modest but statistically significant improvement in OS was shown for the FOLFOX-B arm (12.9 mo *vs* 10.8 mo, $P = 0.0024$). Single agent bevacizumab showed virtually no effect. Immediately after the release of this study, and in spite of the lack of evidence in the front line therapy setting, FOLFOX-B was rapidly accepted in the oncology community as a valid front line option for stage Ⅳ colon cancer. Evidence to support this practice finally materialized in 2008. The NO16966 study was a

Table 1 Selected phase 3 clinical trials involving anti-angiogenic drugs in combination with conventional chemotherapy

Ref.	Drug and study name	Study description	No. of patients	Comparison	Median OS (mo)	Median TTP/PFS (mo)	ORR	1-yr survival
	Bevacizumab (B)							
Hurwitz *et al*[8] 2004	AVF2107g trial	RCT, 1st line	813 (ITT)	IFL + B *vs* IFL	20.3 *vs* 15.6	10.6 *vs* 6.2	45% *vs* 35%	74% *vs* 63%
Fuchs *et al*[10] 2007	BICC-C trial	RCT, 1st line	117 (2nd period)	FOLFIRI + B *vs* mIFL + B	28 *vs* 19	11 *vs* 8	58% *vs* 53%	87% *vs* 61%
Giantonio *et al*[12] 2007	ECOG 3200 trial	RCT, 2nd line post irinotecan 1st line	820 (ITT)	FOLFOX-4 + B *vs* FOLFOX-4 *vs* B alone	12.9 *vs* 10.8 *vs* 10.2	7.3 *vs* 4.7 *vs* 2.7	23% *vs* 8.6% *vs* 3.3%	56% *vs* 43% *vs* 44%
Saltz *et al*[13] 2008	NO16966 trial	RCT, phase 3, 1st line, factorial 2 x 2	1401	FOLFOX-4 or XELOX + B *vs* FOLFOX-4 or XELOX	21.3 *vs* 19.9	9.4 *vs* 8.0	47% *vs* 49%	Not reported
Tebbutt *et al*[17] 2010	MAX trial	RCT, open label, 1st line	471	Cape alone *vs* Cape + B *vs* Cape + B + mitomycin	18.9 *vs* 18.9 *vs* 16.4	5.7 *vs* 8.5 *vs* 8.4	30% *vs* 38% *vs* 46%	Not reported
Cunningham *et al*[18] 2013	AVEX trial	RCT, elder population, 1st line	280	Cape alone *vs* Cape + B	20.7 *vs* 16.8	9.1 *vs* 5.1	19% *vs* 10%	74% *vs* 44%
Falcone *et al*[21] 2013	TRIBE trial	RCT, 1st line	508	FOLFOXIRI-B *vs* FOLFIRI-B	31.0 *vs* 25.8	12.1 *vs* 9.7	65% *vs* 53%	Not reported
Bennouna *et al*[66] 2013	ML 18147	RCT, open label, 2nd line post chemo + B	409	2nd line chemotherapy + B *vs* 2nd line chemotherapy	11.2 *vs* 9.8	5.7 *vs* 4.1	5.5% *vs* 4%	Not reported (approximately 50% *vs* 40%)
	Ziv-Aflibercept							
Van Cutsem *et al*[29] 2012	VELOUR trial	RCT, 2nd line post oxaliplatin and/or bevacizumab 1st line	1226	FOLFIRI + aflibercept *vs* FOLFIRI + placebo	13.5 *vs* 12.0	6.9 *vs* 4.7	20% *vs* 11%	56% *vs* 50%

RCT: Randomized controlled trial; OS: Overall survival; TTP: Time to progression; PFS: Progression free survival; ITT: Intention to treat; ORR: Overall response rate.

non-inferiority trial evaluating the use of XELOX and FOLFOX with or without bevacizumab in a factorial design[13]. The primary analysis demonstrated a statistically significant benefit in terms of progression-free survival (PFS) (9.4 mo *vs* 8.0 mo; $P = 0.002$) in patients receiving bevacizumab, irrespectively of the chemotherapy backbone used, but there was no difference in terms of OS and ORR in the final analysis. Moreover, the TREE studies evaluated the use of three different oxaliplatin-based chemotherapies with bevacizumab[14]. A total of 150 patients were randomly assigned to mFOLFOX-6, bFOL (bolus FU and low-dose LV with oxaliplatin) or CapeOx in the TREE-1 cohort and 223 patients were randomized to the same regimens with bevacizumab in the TREE-2 cohort. ORR was superior in each arm with the addition of bevacizumab and, although not statistically significant, it was highest with mFOLFOX-6 and bevacizumab (52%). Additionally, the BEAT study was designed to evaluate the safety and efficacy of several regimens containing bevacizumab used in the daily community practice but outside the formalities of a clinical trial and in a no-comparative fashion[15]. Consistent with previous studies, improved PFS and OS were seen in patients receiving doublet regimens compared to single agent chemotherapy.

A very relevant issue, however, for the daily practice is the fact that many patients with metastatic colon cancer are not suitable (*e.g.*, elder population or poor performance status) to receive multi-agents regimen such as FOLFOX or FOLFIRI. A common practice in these cases is to use single agent fluoropyrimidine (*e.g.*, weekly bolus 5-FU). Even in this situation, there is enough evidence to support the use of bevacizumab. At least one phase II clinical trial proved that the addition of bevacizumab to single agent 5-FU resulted in better PFS (9.2 mo *vs* 5.5 mo, $P < 0.001$) when used as first line option[16]. Importantly, the mean age of the participants was more than 70 years old. Further evidence supporting the efficacy of this combination, especially in fragile patients, came from the MAX study where capecitabine and bevacizumab resulted in longer PFS compared to single agent capecitabine (8.5 mo *vs* 5.7 mo; $P < 0.001$)[17]. This was confirmed by the AVEX Trial that enrolled elder patients (> 70 years) who were not candidates for treatment with oxaliplatin or irinotecan and randomized them to capecitabine alone or in combination with bevacizumab[18]. With a mean follow up close to 2 years, the median PFS was almost double with bevacizumab (9.1 mo *vs* 5.1 mo; $P < 0.001$). ORR was also superior but the study was underpowered to detect a benefit in OS. However, the reader should be aware that the addition of bevacizumab in these three trials resulted in an absolute increment of grade 3-4 toxicity of about 15%-20% with none of them showing a statistically benefit in OS.

A classical paradigm that has been recently called into challenge is the one that discourage the use of multi-agents regimens combining oxaliplatin and irinotecan at the same

time. This presumption was based on the results of the N9741 study where the IROX (oxaliplatin + irinotecan) arm showed worse TTP, ORR and OS compare to FOLFOX[19]. However, treatment with the combination of 48-h infusional 5-FU, oxaliplatin and irinotecan (FOLFOXIRI) proved to be superior to FOLFIRI, which is believed to be similar to FOLFOX, in terms of OS, PFS and ORR in patients with mCC[20]. Recently, the results of a phase 3 TRIBE trial that compared FOLFOXIRI and FOLFIRI with the addition of bevacizumab were presented[21]. Both treatments were administered for a maximum of 12 cycles followed by 5-FU + bevacizumab until progression. With a mean follow-up of 26.6 mo, significantly increased PFS was observed in the FOLFOXIRI-B arm (9.7 mo *vs* 12.2 mo, $P = 0.001$). As expected, greater neutropenia, diarrhea, stomatitis and neurotoxicity were seen in the FOLFOXIRI arm. Interesting, similar results were obtained in a recent randomized phase Ⅱ study (OLIVIA) where FOLFOXIRI-B showed better ORR and conversion to R0 resections compared to FOLFOX-B[22]. Data is still immature, but this combination could be a feasible option for fit patients.

To summarize we should emphasize some useful concepts. First, single agent bevacizumab has almost no activity. Second, the best evidence comes from its usage as upfront first line therapy in combination with either FOLFOX or FOLFIRI and perhaps FOLFOXIRI. In all cases, bevacizumab has persistently showed to improve PFS. For second line treatment the ideal scenario would be in patient who did not receive bevacizumab as a first line option. Lastly, continuation beyond progression is also feasible (see below).

Ziv-aflibercept

Ziv-aflibercept (Zaltrap®, Regeneron Pharmaceuticals) is a recombinant fusion protein consisting of the extracellular domains of human VEGFR-1 and 2 fused to the Fc portion of human IgG-1[23]. The decoy protein binds tightly PIGF, VEGF-A and VEGF-B preventing the activation of VEGFR-1 and 2 by these ligands. This is a significant difference with bevacizumab which exclusively blocks the VEGF-A[24]. Pre-clinical studies confirmed that when combined with cytotoxic drugs, ziv-aflibercept exerted considerable inhibition of angiogenesis[25-27]. In 2006, 38 patients were enrolled in a phase I clinical trial were 2, 4, 5 and 6 mg/kg escalating doses of ziv-aflibercept were explored in combination with irinotecan, 5-FU and leucovorin[28]. In the phase 3 VELOUR trial, patients with metastatic colon cancer but previously treated with oxaliplatin-containing regimens were randomly assigned to receive FOLFIRI with or without ziv-aflibercept every 2 wk[29]. Patients could not have received irinotecan before but up to 30% of them received bevacizumab as front line therapy. The ORR (11.1% *vs* 19.8%, $P < 0.001$), PFS (6.9 mo *vs* 4.6 mo, $P < 0.001$) and OS (13.5 mo *vs* 12.1 mo, $P = 0.003$) were all improved in ziv-aflibercept and were not influenced by the prior use of bevacizumab (stratifying variable). However, the absolute benefit was a modest 1.4 mo in OS.

BLOCKING EGFR AND OTHER KINASES

Cetuximab and panitumumab

In addition of blocking the angiogenesis pathway, another line of investigation that lead to practice-changing outcomes was the one advocated to jamming the EGFR. Once activated, the EGFR triggers a series of downstream phenomenon that ultimately result in tumor growth and survival[30]. It is then simple to understand that blocking EGFR could potentially halt tumor progression. Nevertheless, this basic principle is not always applicable. An overwhelming body of evidence confirmed the futility of blocking the EGFR when downstream molecules are anarchically activated. The strongest evidence comes from the presence of KRAS codons 12 and 13 mutations in exon 2 which virtually turns anti-EGFR strategies useless[31]. But, recent investigations have broadened the number of negative predictive mutations found in the RAS genes family to exons 3 and 4 of KRAS and exons 2, 3 and 4 of NRAS genes[32]. In that sense, testing for KRAS/NRAS mutations could exclude 50% of the patients from an ineffective but potentially harmful therapy. BRAF mutations carry a considerable poor prognosis, but its predictive role is somehow controversial. However, and in spite of this obvious limitation, anti-EGFR therapies have found their place in the treatment of stage Ⅳ colon cancer. Two compounds, cetuximab (Erbitux®, Bristol-Myers) a chimeric monoclonal IgG-1 antibody against EGFR, and panitumumab (Vertibix®, Amgen) a fully humanized monoclonal IgG-2 antibody also directed against EGFR, have received FDA-approval for this indication. Table 2 summarizes the most relevant clinical trials related to these agents.

As part of the pre-clinical investigation, cetuximab was tested in tumor xenografts models and found to have marked synergistic activity with irinotecan, even in previously considered irinotecan-resistant cell lines[33]. This observation was the based for a couple of phase 2 clinical trials which confirmed the clinical utility of cetuximab single agent (approximately 10% ORR) and in combination with irinotecan. However, the first convincing evidence of its clinical utility came from the BOND study where 329 patients with irinotecan-resistant metastatic colon cancer were randomly assigned to either single agent cetuximab (ORR 11%, TTP 1.5 mo) or cetuximab plus irinotecan (ORR 23%, TTP 4.1 mo)[34]. No difference in OS was seen but crossover was allowed. As in the case of cetuximab, single agent panitumumab showed 10% ORR in heavily pretreated patients who formerly received 5-FU, irinotecan and/or oxaliplatin[35,36]. Given the encouraging results as second and third line therapies, it did not take much time until both molecules were tested as first line options. In the CRYSTAL trial, 1217 patients were randomly assigned to FOLFIRI alone or FOLFIRI plus cetuximab as first line treatment[37]. The primary endpoint was PFS and it was statistically prolonged in the cetuximab group, albeit by a modest 1 mo (8.0 mo *vs* 8.9 mo in the whole population and 8.7 mo *vs* 9.9 mo in the KRAS wild-type patients). Cetuximab also resulted in

Table 2 Selected clinical trials involving anti-epidermal growth factor receptor, regorafenib or anti-epidermal growth factor receptor *vs* anti-epidermal growth factor receptor receptor agents

Ref.	Drug and study name	Study description	No. of patients	Comparison	Median OS (mo)	Median TTP/ PFS (mo)	ORR	1-yr survival
	Cetuximab (*C*)							
Cunningham *et al*[34] 2004	BOND trial	RCT, phase 2, 2nd line irinotecan-refractory	329	Irinotecan + C *vs* irinotecan	8.6 *vs* 6.9	4.1 *vs* 1.5	23% *vs* 11%	29% *vs* 32%
Van Cutsem *et al*[37] 2009	CRYSTAL trial	RCT, 1st line	1198	FOLFIRI + C *vs* FOLFIRI	20 *vs* 18.5 and (25 *vs* 21)	9 *vs* 8 and (10 *vs* 8.7)	47% *vs* 39% (59 *vs* 43%)	Not reported (approximately 35% *vs* 25%)
Maughan *et al*[59] 2011	COIN trial	RCT, phase 3, 1st line	729 (KRAS wild type)	Oxaliplatin-based chemo + C *vs* chemo alone	17 *vs* 17.9	8.6 *vs* 8.6	64% *vs* 57%	Not reported
Tveit *et al*[60] 2011	NORDIC VII trial	RCT, open label, 1st line	571	FLOX + C *vs* intermittent FLOX + C *vs* FLOX	19.7 *vs* 20.3 *vs* 20.4	8.3 *vs* 7.3 *vs* 7.9	49% *vs* 47% *vs* 41%	Not reported (approximately 70%)
	Panitumumab (*P*)							
Douillard *et al*[39] 2010	PRIME trial	RCT, phase 3, 1st line	1183	FOLFOX-4 + P *vs* FOLFOX-4	24 *vs* 20 (WT) 15 *vs* 19 (MT)	9.6 *vs* 8 (WT) 7.3 *vs* 8.8 (MT)	55 *vs* 48% (WT) 40 *vs* 40% (MT)	Approximately 75% both (WT) approximately 60% *vs* 75% (MT)
	Regorafenib (*R*)							
Grothey *et al*[47] 2013	CORRECT trial	RCT, phase 3, 3rd line	760	Regorafenib *vs* placebo	6.4 *vs* 5.0	1.9 *vs* 1.7	1.0% *vs* 0.4%	24.3% *vs* 20.0%
	Cetuximab (*C*) *vs Bevacizumab* (*B*)							
Stintzing *et al*[63] 2013	FIRE-3trial	RCT, phase 3, 1st line	592	FOLFIRI + C *vs* FOLFIRI + B	28.7 *vs* 25	10 *vs* 10.3	62 % *vs* 58%	Not reported

RCT: Randomized controlled trial; OS: Overall survival; TTP: Time to progression; PFS: Progression free survival; ITT: Intention to treat; ORR: Overall response rate.

an absolute 8% improvement in ORR (all partial responses) but no benefit in OS was observed. Similar results were reported in a randomized, phase 2 study using FOLFOX instead of FOLFIRI[38]. In this case the ORR was improved by 25% in wild-type patients as it was PFS, but only by 15 d (7.2 mo *vs* 7.7 mo). Interesting, in KRAS mutated patients PFS was actually 3-mo worse in the cetuximab arm. Similarly, in the phase 3 PRIME study, investigators used FOLFOX-4 as the backbone to randomized patients in a 1:1 fashion to panitumumab or placebo[39]. As expected, in the wild-type population ORR (48% *vs* 55%) and PFS (8.0 mo *vs* 9.6 mo) was better with anti-EGFR therapy but in KRAS mutated cases the effect was neutral or even worse.

An important point to mention at this moment is in reference to the solid evidence against the presumption that combining both anti-angiogenic and anti-EGFR molecules at the same time would results in a synergistic effect. At least two large, randomized, phase 3 clinical trials consistently showed that combining bevacizumab with EGFR inhibitors is actually deleterious. The first of them (PACCE trial) randomly assigned 1053 patients to either oxaliplatin- or irinotecan-based chemotherapy plus bevacizumab but with and without panitumumab as first line treatment for metastatic colon cancer[40]. The primary objective for the oxaliplatin-based arm was extension of PFS and in the irinotecan group was safety analysis. Secondary end points for both groups were ORR, OS and safety. A planned interim analysis for safety and efficacy was conducted at 50% of the events and panitumumab was removed due to significantly decreased PFS [hazzd ratio (HR), 1.44; $P = 0.004$] and increase toxicity independently of the KRAS status. Grade 3 or more adverse events were present in 90% of patients treated with panitumumab. The CAIRO-2 trial reported similar detrimental results of adding cetuximab to oxaliplatin, capecitabine and bevacizumab[41]. The addition of cetuximab significantly decreased median PFS (10.7 *vs* 9.4, $P = 0.01$). A total of 88% of patients discontinued the study, 45% due to tumor progression and 24.5% due to adverse events. A third study, the CALGB 80405, was initially designed to evaluate the use of FOLFOX or FOLFIRI with bevacizumab, cetuximab, or both agents together. In base of the results of the previous studies, the arm combining cetuximab and bevacizumab was closed (NCT00265850).

Regorafenib

The last drug to receive FDA-approval was regorafenib (Stivarga®, Bayer). The compound is an orally available multi-kinase inhibitor with activity against multiple targets in-

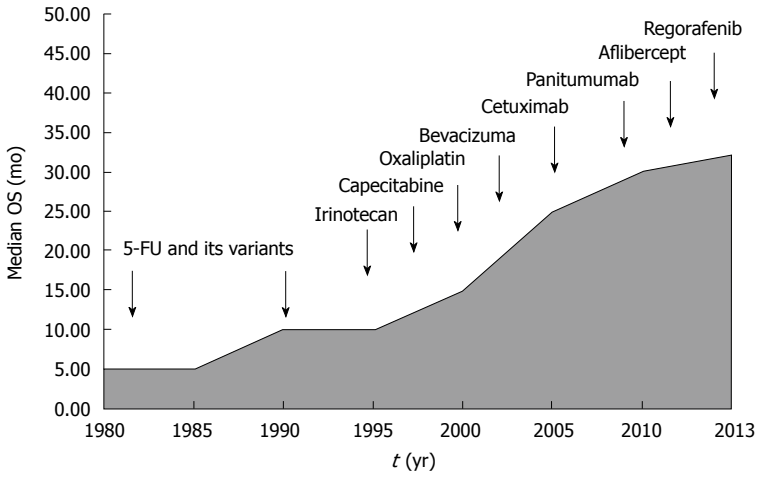

Figure 1 Schematic representation of the recent advances in the treatment of metastatic colon cancer.

cluding KIT, PDGFR and VEGFR among others. It is structurally related to sorafenib and the most usual adverse events are hand-foot skin reaction, mucositis, hypertension and diarrhea[42-45]. In an expanded phase I trial with 27 evaluable patients, 74% achieved disease control with 1 patient obtaining partial response and 19 stable disease[46]. Globally, regorafenib was well tolerated and adverse events were clinically manageable leading to a multi-centric phase 3 trial. The CORRECT study enrolled patients who had already received all the approved standard therapies and who had progressed during or within 3 mo after the last therapy[47]. Seven hundred and sixty participants were randomized in a 2:1 ratio to regorafenib or placebo. Median OS was 6.4 mo in the regorafenib group *vs* 5.0 mo in the placebo group (P = 0.005). The most frequent grade 3 or 4 adverse events were hand-foot skin reaction (17%), fatigue (10%), diarrhea (7%), hypertension (7%), and skin desquamation (6%).

COMMON CLINICAL DILEMMAS

We have witnessed an exponential growth in the number of clinical trials dedicated to metastatic colon cancer which eventually resulted in small but consistent improvement in clinical outcomes (Figure 1). However, this progress has paradoxically leaded us into new challenges. We have arbitrarily chosen 3 topics that in our own opinion are probably the more relevant clinical dilemmas. The reader should be aware, though, that the opinions expressed below come from our own assessment of the literature and they should be considered only as the authors' point of view.

Is there any role for peri-operative chemotherapy in potentially resectable liver metastases? Can the new biological agents improve the resectability rate on patients with borderline or unresectable liver metastases? Which regimen to chose?

The first point to consider is whether the patient has upfront resectable disease or not. A set of criteria have been proposed, however in any case this decision require appropriate discussion between the medical and surgical oncologists[48]. For those who are considered resectable common practice is to give them at least 6 mo of chemotherapy. The most solid evidence for this action comes from the EORTC 40983 trial where 364 patients, with one to four resectable liver metastases, were randomly assigned to surgery alone or 6 doses of FOLFOX-4 pre- and post-surgery[49]. The study was positive for its primary endpoint, PFS (20.9 *vs* 12.5; P = 0.035, per protocol population) and it gained rapid acceptance within the medical community. Oncologist extrapolated these results to the completely neo-adjuvant or adjuvant (stage Ⅳ in NED status) setting, albeit with no evidence to support this approach. OS was not improved in the EORTC 40983 but the enrollment of patients was less than originally expected and its statistical power was called into question. Two other studies were reported in the adjuvant setting after complete resection of liver metastases[50]. They were also underpowered and employed outdated chemotherapy (5-FU bolus). The poor accrual in these clinical trials is most likely related to the oncologists' reluctance to enroll patients in studies that involved a surgery only arm. One single institution, single arm study showed 73% ORR (9% complete pathological response) in 56 patients treated with XELOX + bevacizumab in a peri-operative setting (6 doses pre- and 6 other post-surgery)[51]. The use of biological agents in the post-surgical period, when the patient is NED, is very controversial. Based on the results from adjuvant studies this practice should be discouraged. However, formal studies addressing this issue are missing. Other relevant issue with upfront resectable disease is the fact that chemotherapy could result in liver damage (*e.g.*, steatohepatitis) which could jeopardize patient's only curative chance.

A different scenario presents when the patient has liver-limited but unresectable metastases. Some of these patients (*e.g.*, low volume but abutting critical structures) have borderline disease, potentially amenable to be converted. In these cases, clinician should choose the best possible regimen to obtain maximal response rate. Before

the advent of the anti-EGFR and bevacizumab, conventional chemotherapy agents had already proven to enable surgical resection in a proportion of patients. Regimens such as FOLFOX or FOLFIRI have a conversion rate close to 40% and this could be improved with FOLFOXIRI[20,52,53]. The obvious question then is how much bevacizumab or the anti-EGFR drugs add to this and which one to use. A practical consideration is the fact that bevacizumab, which is the only option in KRAS mutant cases, has to be stopped at least 6-wk before surgery. For wild-type tumors, evidence may be slightly stronger for anti-EGFR drugs.

In the Germanic CELIM phase 2 study, 114 patients were randomly assigned to FOLFOX-6 or FOLFIRI, both regimens with cetuximab[54]. Patients required having technically unresectable liver metastases or more than five lesions. From a 106 evaluable patients, 36 of them (34%) had R0 resection but this proportion reached 60% in the wild-type KRAS population (41/68). Similar results were obtained in retrospective series. Even stronger evidence supporting the use of anti-EGFR in this particular setting came from a recently published Chinese study[55]. This phase 2, randomized study compared the efficacy of conventional chemotherapy (FOLFOX-6 or FOLFIRI) with or without cetuximab. Conversion to resection was the main outcome and after randomizing 138 patients the arm with cetuximab duplicated the proportion of patients deemed eligible for resection (13% *vs* 29%) and triplicated the R0 rates (7.4% *vs* 25.7%). Based on these reports chemotherapy plus cetuximab should be strongly considered for patients with wild-type KRAS and liver only metastases. Detractors of this posture may argue, though, that in a fresh head-to-head comparison between cetuximab and bevacizumab, ORR was not different (FIRE-3; see below).

Data supporting the use of bevacizumab in this scenario is somehow controversial. The most vigorous argument against its use comes from the previously mentioned NO16966 study[14]. There was no difference in ORR and there was similar proportion of patients attempted to have curative metastatectomies (8.4% *vs* 6.0%). However, the study was not designed to test this hypothesis. On the other hand, small phase 2 and retrospective studies brought up to 40% conversion rates and pathological responses when bevacizumab is added to XELOX, representing the fundaments for its use especially in KRAS mutant patients[56,57]. In that regards, the possibility of adding a stronger chemotherapy, such as FOLFOXIRI, should be seriously considered for fit patients.

Which is the ideal chemotherapy mate of the current monoclonal antibodies? And in patients with wild-type KRAS which strategy we should choose? Anti-VEGFR or Anti-EGFR?

Doublet chemotherapy is often used as upfront systemic treatment for advanced CC. It is unclear to these days which doublet is better for each patient and this has to be individualized according to toxicity and comorbidities. FOLFOX, XELOX, and FOLFIRI appear to be similar in efficacy but with different toxicity profile. XELIRI is harder to endure. Most patients tolerate a chemotherapy doublet, but probably not all of them need it as showed by the frequently forgotten Dutch study (CAIRO-1)[58]. The addition of biologics has improved outcomes, but not as much as we hoped. When KRAS is mutated, the chemotherapy chosen must be accompanied with bevacizumab. The dilemma starts with the K-RAS wild type patients. There are clinical trials showing benefit for both approaches: anti-VEGFR and anti-EGFR. The question is which patient would benefit from one or the other schema.

As previously mentioned, in the NO16966 study bevacizumab extended PFS by 1.4 mo, with a more profound effect seen in the XELOX arm[13]. But, why bevacizumab had such a discrete effect on PFS? Was this due to no synergistic or additive effect with FOLFOX/XELOX? The answer is NO, since FOLXOX + bevacizumab is active, even in second line with significant prolongation of OS[12]. Some authors advocate the idea of failure due to the "OPTIMOX" effect, meaning when neurotoxicity occurred oxaliplatin was stopped and fluoropyrimidine plus bevacizumab was continued until progression. This could be the case, since when we observe the difference in PFS of the patients on treatment, this is much more important. It is also feasible that bevacizumab works better with "inferior chemotherapies" such as IFL and have less to offer with "superior chemotherapies" such as XELOX or FOLFOX.

Regarding the anti-EGFR therapies, the earlier cited CRYSTAL and PRIME studies are the foundations for its use in the frontline treatment[40,41]. Nonetheless, in 2011 the COIN study was published[59]. With 2445 KRAS wild-type patients randomized to XELOX or FOLFOX +/- cetuximab, the COIN study represents the biggest trial ever conducted in this population. The results were disappointing. No difference in PFS was seen. Shortly thereafter, the results of the NORDIC VII were released[60]. Patients were randomly assigned to either standard Nordic FLOX or cetuximab + FLOX or cetuximab + intermittent FLOX. The median PFS was 7.9, 8.3, and 7.3 mo respectively and was not significantly different. In patients with KRAS wild-type tumors, cetuximab did not provide any additional benefit but in patients with KRAS mutations a trend toward worsening PFS was observed. The authors concluded that cetuximab did not add significant benefit to the Nordic FLOX regimen as first-line treatment. Additionally, the randomized, phase 2, PEAK study was presented in the 2013 ASCO GI Meeting[61]. This study enrolled 285 patients and evaluated the use of first-line mFOLFOX-6 + panitumumab *vs* bevacizumab. Again, no difference was observed. It is confusing how to interpret the actual role of anti-EGFR and chemotherapy since COIN, the largest phase 3 randomized trial, was negative. The NORDIC was a negative trial as well, but in the scenario of 5-FU given by bolus, a seldom used strategy nowadays.

It is possible that irinotecan-based chemotherapy would be necessary when anti-EGFR is considered in the treatment of metastatic disease. It is also curious that the hazard ratios for PFS with anti-EGFR antibodies tend to become more significant as the number of previously used lines of treatment upsurges. For instance, these agents are useless in the adjuvant setting and grow more active as disease progresses (*e.g.*, 3rd line).

Lastly, the FIRE-3 trial was presented in June 2013[62]. This was a randomized multicenter trial comparing the efficacy of FOLFIRI + cetuximab *vs* FOLFIRI + bevacizumab in patients with wild-type KRAS metastatic colon cancer. The primary endpoint was ORR and 592 patients were included. The study was negative for its primary end-point, with comparable ORR (62% *vs* 58%, $P = 0.183$). Significantly better PFS and OS were seen in the FOLFIRI + cetuximab arm (28.8 mo *vs* 25.0 mo; $P = 0.016$) although this was a secondary endpoint. A preplanned analysis of the FIRE-3 was presented at the European Cancer Congress 2013, aimed to investigate the effect of several other mutations beyond the exon 2 as well as BRAF (V600E)[63]. About 15% of patients were found to have these extra mutations. This sub-analysis incorporated 342 KRAS wild-type patients and 178 KRAS mutant patients (113 with exon 2 mutations plus the 65 newly identified patients). The subgroups were compared for ORR, PFS, and OS. Wild-type patients had 33.1 mo OS with FOLFIRI + cetuximab in comparison to 25.6 mo with FOLFIRI + bevacizumab (HR = 0.70; $P = 0.011$). In KRAS-mutant patients, this difference was not observed. No difference in PFS was seen in the KRAS wild-type group ($P = 0.54$), but interestingly for KRAS-mutated patients PFS was better in the bevacizumab arm (12.2 mo *vs* 6.1 mo; $P = 0.004$). ORR was similar between the arms, irrespective of KRAS status. It is difficult to understand why a treatment that does not improve ORR and PFS could show such an impact on OS.

In conclusion, in 2014 we have only one approach for KRAS mutated tumors which is chemotherapy plus bevacizumab. For KRAS wild type we can use either chemotherapy plus anti-EGFR antibodies OR chemotherapy plus bevacizumab. Going deeply into this last category, at least one clinical trial suggested cetuximab + FOLFIRI as the possible best option. However, head-to-head comparison with FOLFOX+B is lacking and this still represents a valid option. We disfavor oxaliplatin-based chemotherapy with cetuximab based on the MRC COIN study.

Which is the best strategy after progression with bevacizumab-containing regimen? Switch chemotherapy and keep anti-VEGFR or switch to anti-EGFR antibodies?

Preclinical data showed that continuous VEGF inhibition prevents tumor regression[64]. However, risk-benefit ratio associated with continuing bevacizumab use after initial progressive disease was unknown. In 2008, Grothey *et al*[65] reported a novel observation gathered from the BRiTE study. In this large, observational cohort study patients were classified according to the treatment received once they progressed to first line bevacizumab containing regimens. Three groups were identified; those with no post-progression treatment, those who received no-bevacizumab related treatment and those who continued bevacizumab beyond progression. When adjusted for other variables, bevacizumab beyond progression was associated with longer survival ($P < 0.001$). Based on the hypothesis generated by the BRiTE investigators, a randomized phase Ⅲ study-ML18147 trial-was launched[66]. The investigators assessed continuation bevacizumab plus second-line chemotherapy (no anti-EGFR) after standard first-line bevacizumab-based treatment. Bevacizumab lead to a 1.4 mo longer OS (11.2 mo *vs* 9.8 mo; $P = 0.006$).

At the present time is unclear how to proceed in patients who are treated with bevacizumab-containing chemotherapy who progress. In the KRAS/NRAS mutated patients the concept is to maintain the anti-angiogenic status in a similar strategy as the one employed in HER-2/Neu positive breast cancers[67]. This could be achieved either by keeping bevacizumab and changing the chemotherapy regimen or by switching to ziv-aflibercept and irinotecan containing regimen. For wild type tumors, the same options applied but anti-EGFR monoclonal antibodies should be strongly considered because it is important to emphasize that independently of the biological agent chosen first, once progressed patients with wild type tumor should be able to receive all agents sequentially[68].

NEW TARGETS

In the previous sections we have focused on the evidence behind what is currently considered the state of the art treatment of metastatic colon cancer. However, since this field is quite dynamic and the frontiers are in continuous expansion, it will be appropriate to discuss some of the new strategies that are currently being investigated. For description purposes, we will subdivide them based on its main mechanism of action.

Intracellular anti-EGFR therapies

Monoclonal antibodies block the extracellular domain of EGFR. Tyrosine kinase inhibitors (*e.g.*, erlotinib or gefitinib) target the intracellular domain of the receptor. Unlike lung cancer, EGFR mutations are rarely found in colon cancer and are usually not associated with response[69]. Moreover, positive EGFR protein expression does not predict response to treatment[70]. Results have been generally disappointing with no objective responses seen with erlotinib and no improvement in OS with the combination of gefitinib and FOLFIRI[71,72]. However, and after many previous unsatisfactory attempts, a positive study was finally published. Tournigand and colleagues recently presented the results of the phase 3 DREAM trial (OPTIMOX Ⅲ) showing that the addition of erlotinib to bevacizumab maintenance therapy after induction with chemotherapy + bevacizumab resulted in a small, but statistically

significant improvement in PFS from 4.6 to 5.8 mo (P = 0.005)[73]. Remarkably, KRAS mutation status was not a determinant of efficacy and patients with KRAS mutated had even better results. Some clinical trials are currently assessing the role of dual EGFR blocking (panitumumab + erlotinib) with or without chemotherapy in patients with progressed KRAS wild type tumors (NCT00940316). This approach is attractive especially in patients with poor performance status. Nonetheless, it will be at least 1 or 2 years before results become available.

BRAF inhibitors

Vemurafenib targets the BRAF V600E mutation and was proved to be effective in advanced melanomas. Unfortunately, results have been elusive in stage Ⅳ colon cancer. In a small phase I study in patients with BRAF mutant metastatic disease, only 1 of 19 patients had a partial response with single agent vemurafenib[74]. Apparently, blocking the BRAF pathway causes a reflective hyperactivation of the EGFR pathway. For that reason, there seems to be some rationale in combining BRAF and EGFR inhibitors and in preclinical studies a synergistic effect was found[75]. An ongoing trial is evaluating the combination of vemurafenib and cetuximab (EUDRACT # 2011-004426-10).

Pi3K pathway

PTEN loss has been associated with worse survival outcomes in colon cancer[76]. Some studies have also shown that PIK3CA mutations and PTEN loss are associated with an absence of response to anti-EGFR therapies[77]. Aspirin seems to be able to block the PI3K pathway. In a recent retrospective study only patients with PIK3CA mutant but not wild-type colorectal cancers who took daily aspirin had better cancer-specific and OS than those who did not take aspirin[78]. A phase 2 trial combined capecitabine plus perifosine (an inhibitor of the PI3K/Akt/mTOR pathway) with promising activity; however the phase 3 was negative[79]. Additionally, the combination of MEK and PI3K/mTOR inhibitors is currently being evaluated in a phase 1 trial (NCT 01390818) and Hochster *et al*[80] recently reported stimulating results with the combination of selumetinib (MEK inhibitor) and irinotecan.

HER-2 pathway

Few studies, with inconsistent results, investigated the role of HER-2 gene amplification as a potential predictive factor for anti-HER2 therapy. Some reported that HER-2 amplification was associated with resistance to cetuximab and worse PFS or OS; others found neither predictive nor prognostic value in HER-2[81-82]. A phase 2 study evaluating the combination of FOLFOX and trastuzumab in patients who have progressed after 5-FU and/or irinotecan-containing therapy was recently concluded; results are pending (NCT00006015).

Antiangiogenics

In addition to bevacizumab and ziv-aflibercept, other anti-angiogenic drugs have been evaluated with mixed results. Cediranib, a VEGFR inhibitor, showed comparable efficacy to bevacizumab but was associated with increased toxicity[83]. A dual EGFR and VEGFR inhibitor, vandetanib, was ineffective[84]. Ramucirumab, an anti-VEGFR-2 monoclonal antibody, is currently under evaluation in a phase 3 (NCT01183780) following promising results in a phase 2 study[85]. Since there is no real validated marker to predict response to anti-angiogenic drugs, it may take some time before any other anti-angiogenic compound make it to the market.

Insulin growth factor axis

The insulin growth factor (IGF) cascade activates a number of intracellular signaling pathways, including the Ras/Raf/MAPK pathway and the PI3K/Akt pathway[86]. Consequently, it is a potential target for a number of drugs. The main drugs developed as IGF inhibitors have been monoclonal antibodies. Dalotuzumab failed at an interim analysis of a phase 2/3 trial but pre-specified biomarker analysis suggested that patients with higher levels of IGF-1 may be a small subgroup who would potentially benefit from this treatment. Consequently, this hypothesis is being evaluated in a phase 2 study (NCT01609231).

Immunotherapy

In spite of the tremendous excitement raised by innovative immune-therapies in other solid tumors the scenario in metastatic colon cancer has been quite frustrating. No responses were seen in early phase trials with ipilimumab[87]. The same occurred with anti-PD-1 antibodies[88]. Currently, some investigators are testing the use of vaccines (NCT01322815). However, colon cancer seems to remain indifferent against this immunological "rush" or "fever" that we are living at this moment.

CONCLUSION

In conclusion we can affirm that over the last couple of years we have made some small but consistent progress against colon cancer. Anti-angiogenic and anti-EGFR strategies have given dividends by prolonging PFS and to a lesser extend prolonging life in patients with metastatic disease. We are still learning how to use them and it may take time before we discover the best sequence and combination. We also expect that in the near future better biomarkers lead us to the deeply desire but still evasive personalized medicine. But beyond these small victories, new horizons are envisioned. For example, half of the patients have KRAS/NRAS mutant tumors, though there are few drugs that target RAS directly. However, bypassing agents such as MEK inhibitors either alone or in combination with PI3K inhibitors may show promising results. It is impossible to predict the future, but it is expectable and even desirable that soon this review will become obsolete. That is human nature. That is progress. And that is why we must force ourselves to keep us continuously updated.

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ORIGINAL ARTICLE

Novel diet-related mouse model of colon cancer parallels human colon cancer

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Abstract

AIM: To investigate the close parallels between our novel diet-related mouse model of colon cancer and human colon cancer.

METHODS: Twenty-two wild-type female mice (ages 6-8 wk) were fed the standard control diet (AIN-93G) and an additional 22 female mice (ages 6-8 wk) were fed the control diet supplemented with 0.2% deoxycholic acid [diet + deoxycholic acid (DOC)] for 10 mo. Tumors occurred in the colons of mice fed diet + DOC and showed progression to colon cancer [adenocarcinoma (AC)]. This progression is through the stages of tubular adenoma (TA), TA with high grade dysplasia or adenoma with sessile serrated morphology, intramucosal AC, AC stage T1, and AC stage T2. The mouse tumors were compared to human tumors at the same stages by histopathological analysis. Sections of the small and large intestines of mice and humans were evaluated for glandular architecture, cellular and nuclear morphology including cellular orientation, cellular and nuclear atypia, pleomorphism, mitotic activity, frequency of goblet cells, crypt architecture, ulceration, penetration of crypts through the muscularis mucosa and presence of malignant crypts in the muscularis propria. In addition, preserved colonic tissues from genetically similar male mice, obtained from a prior experiment, were analyzed by immunohistochemistry. The male mice had been fed the control diet or diet + DOC. Four molecular markers were evaluated: 8-OH-dG, DNA repair protein ERCC1, autophagy protein beclin-1 and the nuclear location of beta-catenin in the stem cell region of crypts. Also, male mice fed diet + DOC plus 0.007% chlorogenic acid (diet + DOC + CGA) were evaluated for ERCC1, beclin-1 and nuclear location of beta-catenin.

RESULTS: Humans with high levels of diet-related DOC in their colons are at a substantially increased risk of developing colon cancer. The mice fed diet + DOC had levels of DOC in their colons comparable to that of humans on a high fat diet. The 22 mice without added DOC in their diet had no colonic tumors while 20 of the 22 mice (91%) fed diet + DOC developed colonic tumors. Furthermore, the tumors in 10 of these mice (45% of mice) included an adenocarcinoma. All mice were free of cancers of the small intestine. Histopathologically, the colonic tumor types in the mice were virtually identical to those in humans. In humans, characteristic aberrant changes in molecular markers can

be detected both in field defects surrounding cancers (from which cancers arise) and within cancers. In the colonic tissues of mice fed diet + DOC similar changes in biomarkers appeared to occur. Thus, 8-OH-dG was increased, DNA repair protein ERCC1 was decreased, autophagy protein beclin-1 was increased and, in the stem cell region at the base of crypts there was substantial nuclear localization of beta-catenin as well as increased cytoplasmic beta-catenin. However, in mice fed diet + DOC + CGA (with reduced frequency of cancer) and evaluated for ERCC1, beclin-1, and beta-catenin in the stem cell region of crypts, mouse tissue showed amelioration of the aberrancies, suggesting that chlorogenic acid is protective at the molecular level against colon cancer. This is the first diet-related model of colon cancer that closely parallels human progression to colon cancer, both at the histomorphological level as well as in its molecular profile.

CONCLUSION: The diet-related mouse model of colon cancer parallels progression to colon cancer in humans, and should be uniquely useful in model studies of prevention and therapeutics.

Key words: Diet; Deoxycholate; Mouse model; Colon cancer; Histology; Chlorogenic acid; 8-OH-dG; Beclin 1; Beta-catenin

Core tip: Mouse models of colon carcinogenesis are essential as platforms for trials of prevention and therapy. However, most previous rodent models of colon carcinogenesis lack an invasive phenotype and/or do not share several significant genetic events and histopathological features of human colon cancer. This new diet-related mouse model of colon cancer is unique in being closely parallel to human progression to sporadic colon cancer by measures of its histomorphology and its molecular profile. It also has a natural basis, using dietary deoxycholic acid, long thought to be a central causative agent in colon carcinogenesis.

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INTRODUCTION

Epidemiological studies show that rates of colon cancer incidence and mortality vary substantially across regions of the world. The rate of colon cancer incidence differs between countries by more than 10-fold[1]. More dramatically, Native Africans in South Africa have a colon cancer rate of < 1:100000[2] compared to the incidence rate for male African Americans of 72:100000[3]. In populations migrating from low-incidence to high-incidence countries rates change rapidly, and within one generation may reach the rate in the high-incidence country. This has been observed, for instance, in the colon cancer incidence of migrants from Japan to Hawaii[4]. These changes in colon cancer rates are thought to be largely due to changes in diet. Large increases in both meat and fat in the diet correlate with large increases in rate of colon cancer, graphed on an exponential scale[5].

In populations with a high incidence of colorectal cancer, fecal concentrations of bile acids are increased[6,7], suggesting that increased exposure of the colonic lumen to high levels of bile acids plays a role in the natural course of development of colon cancer. For example, the concentration of deoxycholic acid (DOC) in the feces of Native Africans in South Africa is 7.30 nmol/g wet weight stool while that of African Americans is 37.51 nmol/g wet weight stool, so that there is 5.14 fold higher concentration of DOC in stools of African Americans than in Native Africans[8]. As indicated above, there is a more than 72-fold greater rate of colon cancer in African American males than in Native Africans of South Africa. The hydrophobic bile acids, DOC and lithocholic acid, appear to be the most significant bile acids with respect to human colorectal cancer[6].

Since the bile acid DOC was implicated as important in colon cancer etiology in humans, we previously investigated whether DOC, at a high human physiologic level, could be a colon carcinogen in an experimental mouse model[9], and found that a high human physiologic level of DOC in the mouse colon does indeed cause colon cancer. We investigate, in the current study, whether the progression to colon cancer due to high physiologic levels of DOC in the mouse, by the gold standard histomorphologic analysis[10], is closely parallel to progression to colon cancer in humans. Other studies indicate that preneoplastic areas (field defects) are altered in molecular markers in human progression to colon cancer. We evaluate four of these markers: 8-OH-dG, ERCC1, beclin-1 and beta-catenin in the mouse colon progressing to colon cancer.

MATERIALS AND METHODS

Animals

Wild-type female B6.129PF2/J mice, ages 6-8 wk old, were obtained from Jackson Laboratories (Bar Harbor, ME). The mice were the second generation (F2) of a cross between two well-established, inbred, wild-type strains: C57BL/6J and 129S1/SvlmJ (one of which carried a recessive albino mutation). The phenotypes of these F2 wild-type mice is expected to be varied, since the contribution of the two parental wild-type strains will be different in each F2 offspring, as illustrated by the color variation in these mice (Figure 1). It was intended that these mice be similar to a normal healthy human population in their genetic variation. Mice were main-

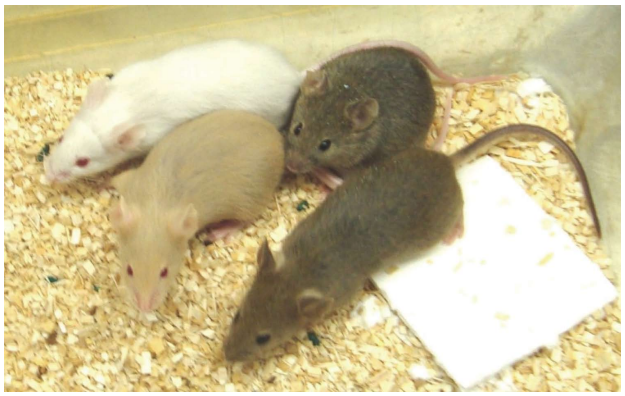

Figure 1 Young mice from 2nd generation cross of 2 wild type inbred lines show variation in colors.

tained at the University of Arizona's Animal Care Facility. All animals were raised, starting with 4 mice in each pan, in cages under nonsterile microisolator conditions and in compliance with the regulations and NIH guidelines for Care and Use of Laboratory Animals. All mice were weighed and their weights recorded weekly.

The mice were free of murine viruses, pathogenic bacteria (including *Helicobacter spp.*), and endo- and ecto-parasites by routine health evaluations. The mice were maintained on a 12-h light-dark cycle with water ad libitum and fed the control AIN-93G diet (Table 1), either unsupplemented or supplemented with 0.2% DOC. Purified diets were prepared as needed by Harlan Teklad, Madison, WI (including the DOC-containing diet). DOC was supplied by Sigma-Aldrich Corp, St. Louis, MO. Mice were first fed the control diet for 2 wk for acclimation. Then half the mice were fed with diet + DOC and half with control diet alone. Ten months after being switched to their experimental diets the mice were sacrificed, using CO_2. At the time of being placed on the experimental diets, 24 mice fed the control diet and 24 mice fed diet + DOC each consisted of 6 mice 6 wk old, 15 mice 7 wk old, and 1 mouse 8 wk old. During the succeeding 10 mo, 2 mice from each group died of unknown causes so that 22 mice in each group completed the experiment.

Histopathology, gross and microscopic images of human tissue

Before any biopsy tissue samples were obtained during colonoscopy, informed consent was given by the patient, using a form approved by the University of Arizona Institutional Review Board. Biopsy specimens were completely fixed in 10% buffered formalin for 6 to 12 h, followed by routine processing through graded alcohols and subsequent embedding into paraffin blocks. Tissue samples from colonic resections were obtained after informed consent before surgery. Colonic segments were cut open and gross photographic images of colonic tumors and polyps were obtained. Adequate representative tissue samples were obtained from areas of tumors and adjacent colonic mucosa. Similar to the biopsy specimens, these tissue samples were fixed in 10% buffered formalin for 24 to 36 h, transferred to graded alcohols, followed by paraffin embedment.

Table 1 AIN-93G diet composition

Ingredients	Percentage
Corn starch	39.75%
Casein vitamin free	20%
Maltodextrin	13.20%
Sucrose	10%
Soybean oil	7%
Powdered cellulose	5%
AIN 93G mineral mix	3.50%
AIN 93 vitamin mix	1%
L-cystine	0.30%
Choline bitartrate	0.25%
t-butylhydroquinone	0.0014%

Three 4-micron tissue sections were cut from all retained paraffin-embedded tissues. The tissues were then placed on glass slides, stained with hematoxylin and eosin, and subjected to histopathologic analyses. Morphologic evaluation was performed using a brightfield digital light microscope (Motic BA300).

Histopathology, gross and microscopic images of mouse tissue

The gastrointestinal (GI) tracts of mice, including rectum, colon, cecum, small intestine, stomach and lower esophagus, were removed, opened longitudinally, rinsed with phosphate-buffered saline (PBS) and divided into sections that could fit into paraffin blocks. All parts of the lower GI tract including rectum, colon and cecum were retained for fixation and paraffin embedment and any parts of the small intestine, stomach and esophagus that had a visible protrusion were retained. In addition, other organs including liver, pancreas, spleen, breasts and lymph nodes near breasts were examined, and if there were any potentially aberrant areas observed, sections of these organs were also retained. All retained sections were placed flat on Matricel membranes for good orientation. Segments of intestine with grossly visible mucosal nodules were photographed with a Sony Cybershot 7.2 megapixel camera. Sections were subsequently fixed in 10% formalin overnight at 4 ℃, then transferred to 70% alcohol, and embedded in paraffin.

Three to six 4-micron tissue sections were cut (multiple sections were cut to ensure any tumors or aberrant areas were included in the sections) from all retained tissues. The tissues were then placed on slides, stained with hematoxylin and eosin, and assessed for histopathologic characteristics. Morphologic evaluation was made on all the tissues on slides, using a brightfield digital microscope (Motic BA300). There is currently no accurate substitute for histopathologic determination of colonic neoplasia[10].

Diagnosis of histopathology

Anil R Prasad, MD, a surgical and cytopathologist with years of experience in GI pathology and immunohistochemistry diagnosed all of the tumors detected on the

basis of histopathologic criteria. The mouse tumors were compared to human tumors at the same stages by histopathological analysis. Sections of the small and large intestines of mice and humans were evaluated for glandular architecture, cellular and nuclear morphology including cellular orientation, cellular and nuclear atypia, nuclear enlargement, hyperchromasia, chromatin clearing, pleomorphism, presence of nucleoli, atypical mitotic activity, frequency of goblet cells, crypt architecture, ulceration, invasion of malignant glands through the muscularis mucosa and submucosa and presence of infiltrating malignant glandular crypts within the muscularis propria. Digital photomicrographs of representative sections were obtained using Motic Images Plus 2.0 software.

Immunohistochemistry

Protein expression was assessed using standard immunohistochemical methods[11,12], with variations as needed, described here. Briefly, formalin-fixed and paraffin-embedded tissues were cut into 4 μm sections and floated on water, the tissue sections were picked up onto slides, deparaffinized, and then rehydrated.

Antigen retrieval for 8-OH-dG was performed by immersing slides in 4 mol/L HCl for 20 min at room temperature, rinsing in distilled water four times, transferring slides to 0.1 mol/L Borax for 5 min at room temperature, rinsing four times in distilled water and placing slides, twice, in PBS, pH 7.4, for 5 min.

For ERCC1, antigen retrieval was performed in citrate buffer (2.1 g citric acid + approximately 5 mL 5 mol/L NaOH + 1 L water, pH 6.1) brought to a boil in a microwave and then kept at high temperature for 6 min in the microwave followed by cooling on ice for 20 min. The slides were then washed with PBS for three minutes followed by a distilled water wash for three minutes.

Antigen retrieval for beclin-1 was performed by heating in a microwave in 0.1 mol/L citrate buffer (pH 6.1) and then cooling to room temperature.

For beta-catenin, antigen retrieval was performed in citrate buffer at pH 6.0, the slides were brought to a boil in a microwave and then kept at high temperature (not boiling) in the microwave for 10 min, followed by cooling on ice for 20 min. The slides were then washed with PBS for three minutes followed by a water wash for three minutes.

The slides were then rinsed with distilled water. Endogenous peroxidase activity was blocked by incubation in 3% hydrogen peroxide in methanol for 30 min, and then the tissue sections were rinsed with distilled water and PBS. Next, slides were placed in Sequenza staining racks (Shandon Sequenza Immunostaining System from Thermo Scientific, Thermo Fisher Scientific Inc., Waltham, MA) and rinsed with PBS.

For 8-OH-dG, a non-specific protein binding blocking step was used. For this, 150 μL 5% normal horse serum in PBS was added to each slide, which was allowed to stand at room temperature for 60 min. Next, without rinsing, 150 μL antibody against 8-OH-dG (QED 12501 from *QED Bioscience Inc.*, San Diego, CA) diluted with 2% BSA in PBS to 2 μg/mL was added to each slide and the slides were kept in the refrigerator at 4 ℃ overnight, followed by rinsing three times with PBS. Then 100 μL biotinylated secondary rabbit anti-mouse antibody (DAKO 0413) was added at a 1:400 dilution in 2% BSA in PBS, followed by incubation for 30 min at room temperature. At this point, Vectastain ABC reagent was prepared according to the manufacturer's instructions, and allowed to stand for 30 min before use. Then slides were rinsed with PBS three times, three drops of Vectastain ABC reagent were added and slides were incubated at room temperature for 30 min, followed by three rinses with PBS.

For ERCC1, 3 drops per slide of "Background Sniper" (from Biocare Mach 3 kit, Biocare Medical, Concord, CA) were added and left for 10 min at room temperature to reduce non-specific staining of background proteins. The ERCC1 slides were rinsed with PBS. Then a primary mouse monoclonal antibody was used (8F1 from Neomarkers, Freemont, CA). The mouse monoclonal antibody was added at 2 μg/mL in 2% BSA/PBS and left to incubate at room temperature for 45 min before three PBS washes. For the secondary antibody, the polyclonal rabbit anti-mouse Dako Biotinylated secondary antibody (E0413, DAKO Corp., Carpinteria, CA) was added at 120 μL/slide at a 1:300 dilution (in 2% BSA/PBS) and incubated for 30 min at room temperature before being rinsed 3 times with PBS. Vectastain Elite avidin-biotin complex method kit PK 6100 (Vector Laboratories, Inc., Burlingame, CA) was then used according to the manufacturer's instructions at 3 drops per slide and incubated at room temperature for 30 min before 2 rinses with PBS.

For beclin-1, to prevent nonspecific binding, the slides were blocked with 1.5% goat serum (Vector Laboratories, Burlingame) and then immunostained using a polyclonal anti beclin-1 antibody from ProSci Inc. (Poway, Calif, United States) at a concentration of 1 μg/mL. Sections were then incubated using a biotinylated antirabbit secondary antibody (Vector Laboratories) and Vectastain Elite ABC (Avidin Biotin Complex) reagent (Vector Laboratories).

For beta-catenin, first blocking serum consisting of 1.5% normal rabbit serum was prepared by adding 30 μL of normal rabbit serum to 2 mL BSA/PBS (prepared as 500 μL 22% BSA in 5 mL PBS) and then 120 μL was added per slide for one hour. Diluted beta catenin antibody (beta-catenin 610153, BD Biosciences San Jose, CA) was prepared by using beta catenin antibody at 250 μg/mL and diluting 6 μL into 1194 μL of 2% BSA in PBS. Without rinsing the slides, this antibody was added at 120 μL per slide for one hour. At this point, Vectastain ABC reagent was prepared according to manufacturer's instructions, and allowed to stand for 30 min before use. Then the secondary antibody was added. This was a 1:400 dilution of DAKO 0413 rabbit anti-mouse biotinylated IgG (5 μL DAKO per 1995 μL 2% BSA in PBS) (DAKO Corp., Carpinteria, CA), 120 μL per slide for 30 min, followed by three rinses with PBS. Then three drops of Vector ABC reagent was added per slide for 30 min, followed by two washes with PBS.

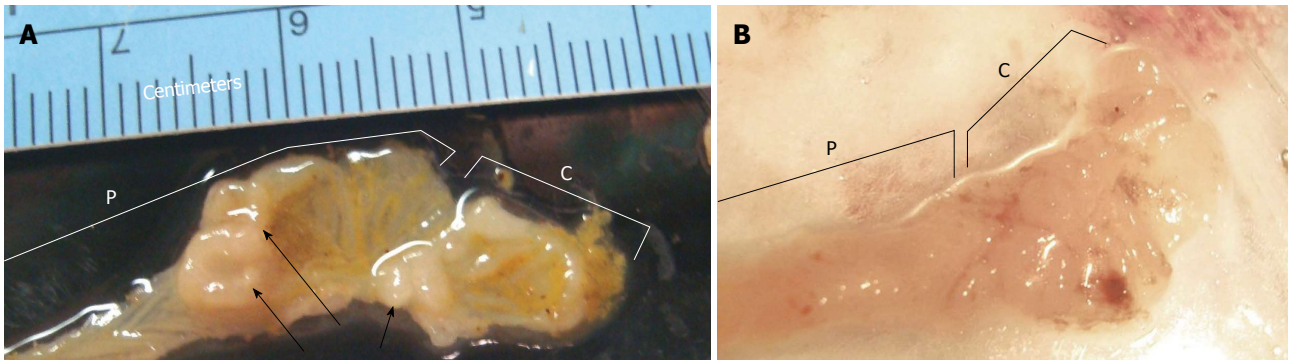

Figure 2 Opened proximal colons plus cecal areas of mice. A: 3 grossly visible mucosal nodules (arrows); B: No visible nodules. The letter P indicates a region of the proximal colon and letter C indicates a cecum.

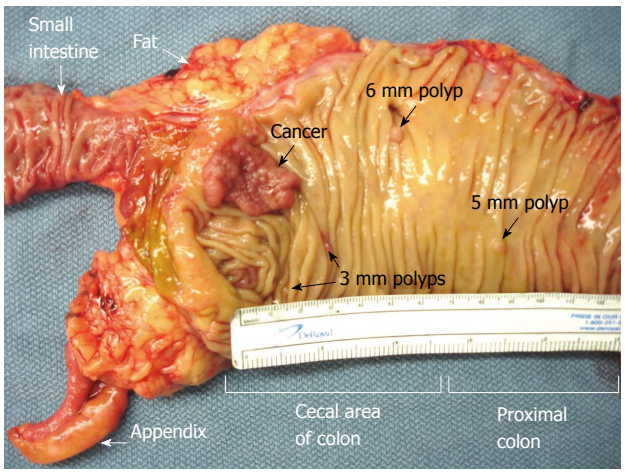

Figure 3 Cut open gross specimen of proximal human colon showing multiple tumors[13].

The slides were then removed from the Sequenzas, and color development was carried out by applying 0.025% diaminobenzidine tetrachloride (Sigma, St. Louis, MO) in PBS supplemented with 0.04% hydrogen peroxide. Sections were counterstained with 1:4 diluted hematoxylin (Sigma), dehydrated in a graded series of ethanols followed by xylene, and then mounted with coverslips using Cytoseal XYL (Richard Allen Scientific, Kalamazoo, MI). Brown staining indicates 8-OH-dG, ERCC1, beclin-1, or beta-catenin expression, and blue staining from hematoxylin identifies nucleoproteins in the nucleus.

Statistical analysis

Because the data was non-normally distributed, the non-parametric Mann-Whitney *U* test was performed to test for differences in occurrence of colonic and duodenal tumors and adenocarcinomas between mice fed diet + DOC and diet alone, and to determine if there were differences in the frequency of proximal and distal colonic tumors in the mice fed diet + DOC. To determine if there were correlations between mouse weight and number of tumors, a Pearson's correlation coefficient was calculated. The statistical analysis package Systat version 12 was used to analyze the data.

RESULTS

Gross physiology of mice fed diet + DOC

Mice fed the control diet and mice fed diet + DOC each looked healthy and were active during the entire time they were on their diets, even though the mice fed diet + DOC were almost all carrying neoplastic lesions (tumors, some of which were cancers) by 10 mo on the diet. This is similar to humans who have colon cancers, who also show no external signs until the cancers are very large or have metastasized.

Macroscopic phenotype of colorectum of mice fed diet + DOC or diet alone

Twenty out of the 22 female mice fed diet + DOC (91%) developed large macroscopically visible mucosal nodules (likely colonic neoplastic lesions). Figure 2 shows opened proximal regions of colons, including the cecums, of two mice fed diet + DOC. Figure 2A shows about 3 cm of proximal colon plus cecum in which three large mucosal nodules can be seen by eye. Histopathological examination of tissue from this area revealed three tubular adenomas, two of them with ulceration and one with high grade dysplasia. Figure 2B shows about 2 cm of another proximal colon plus cecum, and no mucosal nodules are seen. The colon of this mouse, also fed diet + DOC, had no colonic neoplasia at all upon histological examination.

None of the mice fed the control diet alone developed any colorectal tumors, evaluated both macroscopically and by microscopic histopathological examination of all rectum, colon and cecum segments.

Multiple tumors found in one location of the mouse colon, as in Figure 2A, indicate the presence of a field defect. By comparison, in humans, we also found multiple tumors in some of their much larger colon resections, and one example, showing 13 cm of the longitudinally-opened colon, is shown in Figure 3.

Macroscopic phenotype of small intestine of mice fed diet + DOC or diet alone

Most large mucosal nodules seen macroscopically in the

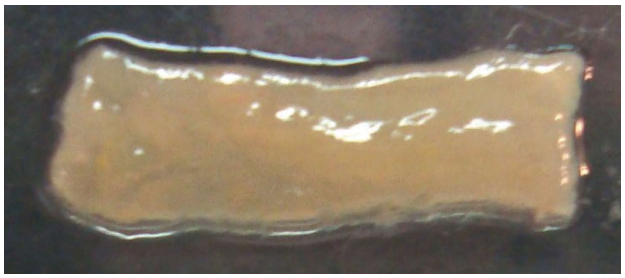

Figure 4 Opened segment of small intestine observed to have mucosal nodules.

large intestines of mice proved to be tumors upon histopathological examination. However, many small mucosal nodules were seen in the small intestine of each mouse, such as shown in Figure 4. Following microscopic examination, almost all were found to be benign Peyer's patches similar to those found in the human small intestine (Peyer's patches are gut-associated lymphoid tissue consisting of isolated or aggregated lymphoid follicles, and are the immune sensors of the intestine).

None of the small mucosal nodules in the small intestines of mice fed diet + DOC proved to be tumors. However, of the 22 mice fed control diet, 3 of the mice had small nodules that proved to be small adenomas. These small adenomas occurred near the Ampulla of Vater and Sphincter of Oddi (at the major duodenal papilla, in the second part of the duodenum), an area that experiences concentrated bile acids as they exit the common bile duct into the small intestine. This is the usual location of small intestinal tumors in humans, as well. These tumors were not cancers.

Types and locations of tumors

For each mouse fed diet + DOC, Table 2 lists data in 11 columns (Note that mice were 6 to 8 wk old when received, acclimated to the control diet for 2 wk, and then put on their diets for 10 mo, so that all mice, at termination, were 12 to $12^{1/2}$ mo old). In column 1, all 22 mice are listed by ascending weights. Columns 2 and 3 give the total number and location (distal or proximal) of all neoplastic lesions in these mice. There were 13 distal and 44 proximal lesions, for a total of 57 lesions.

Columns 4-11 give characteristics associated with the tumors enumerated in columns 2 and 3. Since any particular tumor may have two or more distinguishing characteristics, the total number of characteristics listed is greater than the total number of tumors. Column 4 indicates that two of the tumors in mouse 12 were hyperplastic. Hyperplastic polyps do not exhibit dysplasia and hence do not have malignant potential. Columns 5-8 give the characteristics of polyps exhibiting low and high grade dysplasia. There were 37 with tubular adenoma characteristics (TAs) (column 5), 15 with sessile serrated adenoma characteristics (SSA) (column 6), 17 of these adenomas (TA or SSA) had ulceration (column 7) and 3 adenomas displayed high grade dysplasia (HGD) (column 8). Columns 9-11 indicate characteristics of tumors that contain, or are entirely, clearly malignant and are at an early or later stages. These include 7 intramucosal adenocarcinomas (ACs) (an early stage) (column 9), 9 ACs at stage T1 (column 10) and 2 ACs at stage T2 (a late stage) (column 11). In total, 18 tumors were all, or in part, ACs. The polyps with low and high grade dysplasia (including those with ACs) totaled 55, or an average of 2.5 colonic neoplastic polyps or AC per mouse. The ACs often appreared to arise from a polyp with high grade dysplasia. For example, the mouse weighing 53.7 g had 7 tumors in the proximal colon, and one of these tumors was an SSA from which an AC had arisen and the area of the AC was ulcerated. Overall, 55 tumors were observed displaying morphological characteristics comprised of low and high grade dysplasia, or invasive malignancy of various stages.

Ten of the 22 mice had ACs, with some mice having more than one AC. There were 6 mice having just one AC, 2 mice having two ACs, 1 mouse having 3 ACs and 1 mouse having 4 ACs. Thus 45% of these 22 mice had at least one colonic AC after 10 mo of being fed diet + DOC.

Statistical analysis

As shown in Table 3, after 10 mo on the diet, 20 out of 22 (91%) of mice fed diet + DOC developed tumors (cancers or adenomas) in their colons, and of these diet + DOC fed mice, 10 (45%) had developed cancers. The 22 mice with no supplement to their diet had no cancers or adenomas in their colons. There was a significant difference in the number of mice with colonic tumor development between those mice fed diet + DOC and those fed diet alone (Mann-Whitney *U*, $P < 0.000001$ two-tailed). There was also a significant difference in the number of mice with cancer development between those mice fed diet + DOC and those fed diet alone (Mann-Whitney *U*, $P = 0.00042$ two-tailed).

Of the 57 total tumors found in the mice fed diet + DOC (Table 2), 44 (83%) were found in the proximal colon and 13 (23%) were found in the distal colon. There was a significant difference between the numbers of tumors in the proximal region and the distal region (Mann-Whitney *U*, $P = 0.0027$ two-tailed).

Three of the mice fed the diet only, with no supplement, had small adenomas near the Sphincter of Oddi (at the major duodenal papilla, in the second part of the duodenum). No mice in the DOC + diet group had adenomas in the duodenum. A Mann-Whitney *U* test to determine if there was a significant difference in occurrence of adenomas in the duodenum in the diet + DOC fed mice compared to the mice fed diet alone indicated that there was no significant difference ($P = 0.076$).

Histology of human and mouse colonic tissues compared

Pairs of adjacent images, Figures 5-8 below, illustrate the histomorphology of human and mouse colonic epithe-

Table 2 Mice fed diet + deoxycholic acid

Mouse weights (g)	Locations of tumors		Hyper-plastic polyp	Characteristics of polyps low and high grade dysplasia including those from which cancers arose				Stages of cancers found		
	Distal tumor	Proximal tumor		Tubular adenoma	Sessile serrated adenoma	Ulcerated adenoma	Adenoma with HGD	Intra-mucosal AC	Stage T1 AC	Stage T2 AC
18.7	3			3						
24		3		2			1	1		
25	2			2						
25.8	1				1	1			1	
25.9		3		3		2	1			
26.1		1		1		1				1
26.1	3			3		2			2	
27.3		2		2		1		1		
27.4		5		5		3		3		
28.8		2		2						
35.4	None	None								
35.7	2	3	2	3						
38.9	2	2		2	2					
40		3		2						1
41.1		4			3	3		1	2	
43		2			2	1				
43.1		1		1		1				
45.2		1		1		1	1			
45.2		2		2				1		
49.2	None	None								
53.7		7		3	4	1			4	
78.6		3			3					
Totals	13	44	2	37	15	17	3	7	9	2

AC: Adenocarcinoma; HGD: Highgrade dysplasia.

Table 3 Comparison of diet alone to diet + deoxycholic acid on colonic tumor and cancer development *n* (%)

Diet	Diet (mo)	Mice	Mice with tumors (adenomas + cancers)	Mice with cancer	Tumors (tumor burden[1])	Cancers (cancer burden[2])
Diet alone	10	22	0	0	0 (0)	0 (0)
Diet + DOC	10	22	20 (91%)	10 (45%)	57 (2.6)	18 (0.82)

[1]Tumor burden is the ratio of the number of tumors observed to the number of mice; [2]Cancer burden is the ratio of the number of cancers observed to the number of mice. DOC: Deoxycholic acid.

lial tissues. These Figures identify, in the legends and the images, the specific histomorphological characteristics that are crucial for characterizing either normal glandular architecture or identifiable stages in progression towards invasive adenocarcinoma. Stages shown include normal non-neoplastic glands (crypts) (Figure 5), tubular adenomas (Figure 6), tubular adenomas with high grade dysplasia (Figure 7) and sessile serrated adenomas (Figure 8). In each pair of tissues, the human and mouse crypts show closely parallel specifically identifying histomorphological characteristics. From the microscopic images alone, it is difficult to distinguish whether the tissues are from a human or from a mouse, though when viewed side-by-side, the mouse tissues are seen to have a smaller number of cells per crypt.

Figures 9 and 10 identify, in the legends and the images, the specific histomorphological characteristics that are crucial for characterizing invasive adenocarcinomas of stages T1 and T2. Figure 9A also shows some of the characteristics that may accompany colonic adenocarcinomas. In this image the adenocarcinoma arose in association with, or arose from a sub clone of, a sessile serrated adenoma. In addition, this adenocarcinoma shows ulceration of the colonic mucosa.

Only mouse tissues are shown in Figures 9 and 10, since human adenocarcinomas having penetration through the muscularis mucosa and entry into the submucosa could not be shown at the same magnification and still fit in the figure. These images were taken at intermediate magnification (10× objective lens), a lower magnification than the preceding images (taken with a 40 × objective lens).

Two examples of mouse colonic adenocarcinoma at low magnification (taken with a 4× objective lens) are shown in Figure 10. This magnification allows imaging of the majority of the cancers in single fields of view. Figure 10A shows a section through an entire cancer at stage T1 with mucosal ulceration, and Figure 10B shows a section through an almost entire cancer at stage T2.

Figure 11 shows portions of human and mouse stage

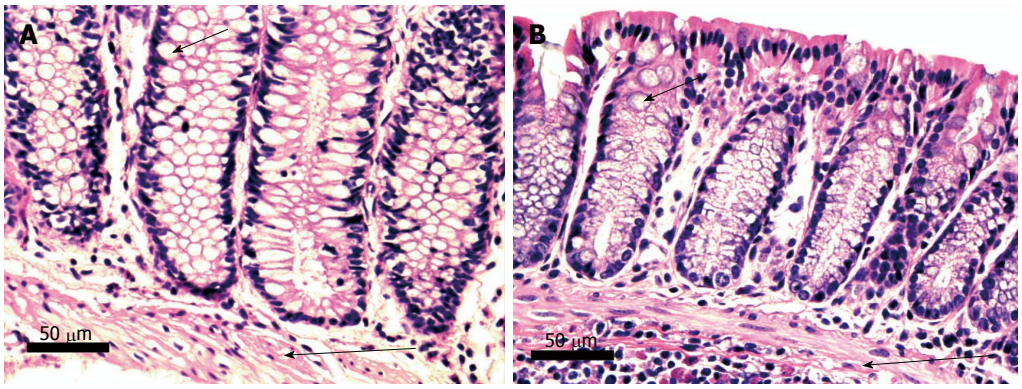

Figure 5 Histologically normal human (A) and mouse (B) colonic crypts, cut along the long axis of crypts. The normal human and mouse glands (crypts) are composed of columnar epithelial cells and goblet cells. Short arrows indicate typical goblet cells containing mucin (not stained, white in the image). About half of the cells in the crypts are goblet cells. Nuclei are darkly stained. All crypts are normally aligned colonic mucosal glands with the bases of the crypts abutting the muscularis mucosa. Long arrows indicate the muscularis mucosa. All crypt cells are parallel to each other and the nuclei are adjacent to each other, with no overlapping. Images obtained with 40× objective lens.

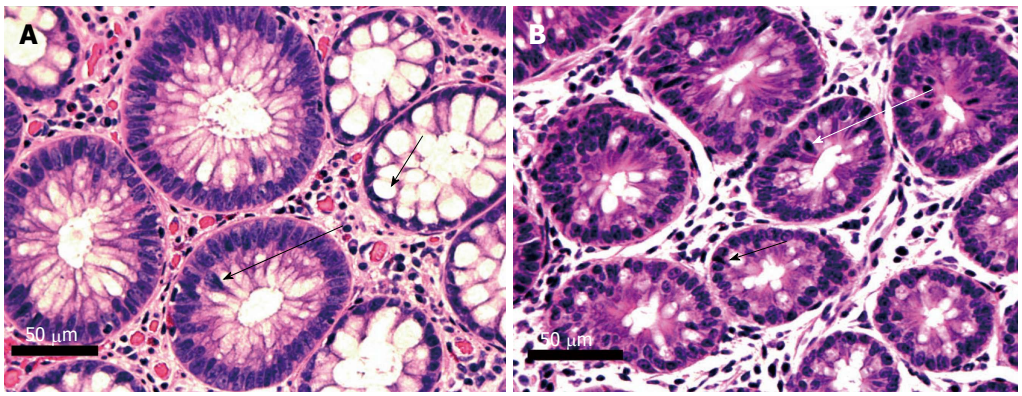

Figure 6 Human (A) and mouse (B) crypts cut across the short axis, showing tubular adenomatous crypts as well as histologically normal crypts. Crypts on the right in A and at the bottom of B have normal histology. Adenomatous crypts are seen to the left in A, and in the top half of B. Adenomatous glands show overlapping cells with hyperchromatic mitotically active nuclei (long arrows indicate examples of cells undergoing mitosis). Short arrows indicate typical goblet cells. The goblet cells in adenomatous glands are decreased in frequency compared to goblet cells in the histologically normal glands. Images obtained with 40× objective lens.

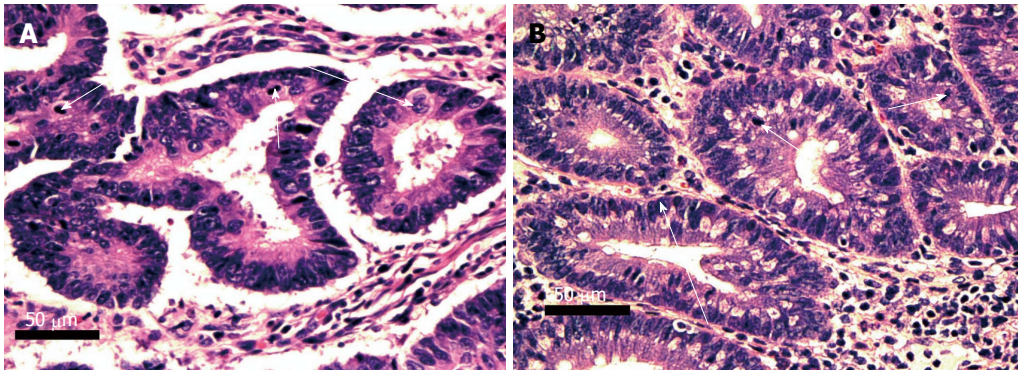

Figure 7 Crypts of tubular adenomas with high grade dysplasia cut across the short axis, human (A) and mouse (B). Glands with high grade dysplasia show overlapping cells with oval to round vesicular nuclei and prominent nucleoli (long arrows). Mitotic figures are abundant (short arrows). Complex architecture with infolding of crypts can also be seen. Images obtained with 40× objective lens.

T2 adenocarcinomas, showing adenomatous glands invading the muscularis propria. The presence of extravasated mucin, forming mucin pools adjacent to malignant glands are seen in Figure 11B.

IHC evaluation of molecular markers for progression to colon cancer

Tissues had been preserved in paraffin from our previous experiment where mice had been fed either the control diet,

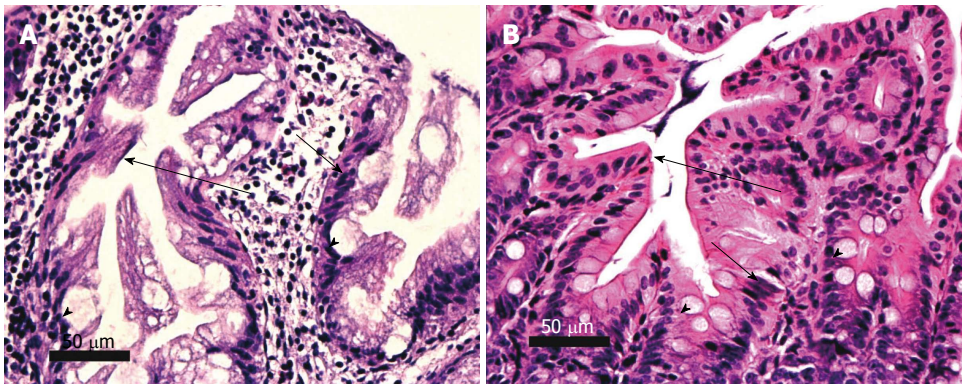

Figure 8 Sessile serrated adenomas, human (A) and mouse (B), cut along the long axis. Serrated glands show star shaped crypt architecture (long arrows). Adenomatous glands with hyperchromatic overlapping nuclei (short arrows) retaining goblet cells (arrow heads) are seen. Images obtained with 40× objective lens.

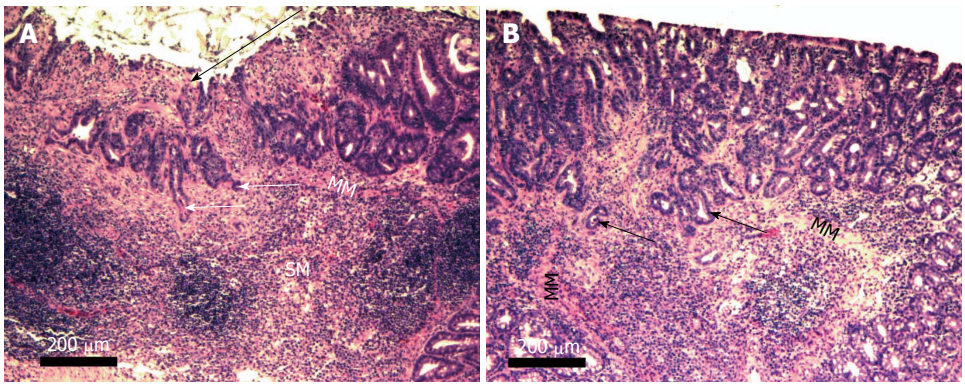

Figure 9 Two examples of mouse adenocarcinoma stage T1. A shows a sessile serrated adenoma in the right upper portion of the image and an ulcerated region (long arrow) above an adenocarcinoma that had penetrated the muscularis mucosa. Both A and B show invasive glands (short arrows) infiltrating through the muscularis mucosa (MM) into the submucosa (SM). Images obtained with 10× objective lens.

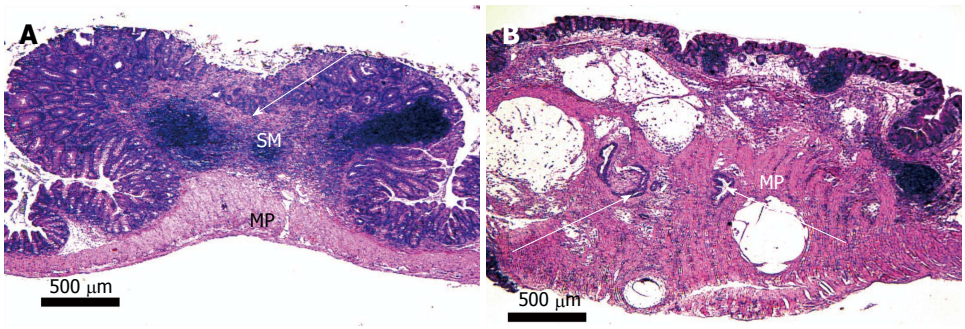

Figure 10 Mouse adenocarcinomas at stages T1 (A) and T2 (B). A shows a section through an entire cancer at stage T1, and B shows a section through an almost entire cancer at stage T2. A shows infiltrating malignant glands (long arrow) in submucosa (SM) but not in muscularis propria (MP). B shows infiltrating malignant glands (long arrows) within muscularis propria (MP). These adenocarcinomas are about 2 to 3 mm tall and about 6 mm wide and would correspond to the sizes of the mucosal nodules seen in Figure 2A. Pale areas in B are pools of mucin. Images obtained with 4× objective lens.

diet + DOC or diet + DOC + CGA[9]. From the colons of each of three mice on the different diets, a 4 micron tissue section was obtained and immunostained for location and level of a marker of progression to colon cancer. The segments of the colons evaluated were in regions of the colon without a neoplastic lesion. Thus, we were evaluating colon segments for the presence of preneoplastic areas from which a neoplastic lesion might be expected to arise. The small number of mouse colons evaluated constituted a brief survey of molecular markers altered in progression to colon cancer. The examples in Figures 12-16 were representative of the levels of biomarkers found, but with only three tissue samples, variation of the expression of each marker was not quantitated. As background information for these tissues, we note that in the previous experiment from which these tissues came, for the 12 mice fed the control diet none developed colonic neoplasia. For the 18 mice fed diet + DOC, 94% had developed colonic neoplasia,

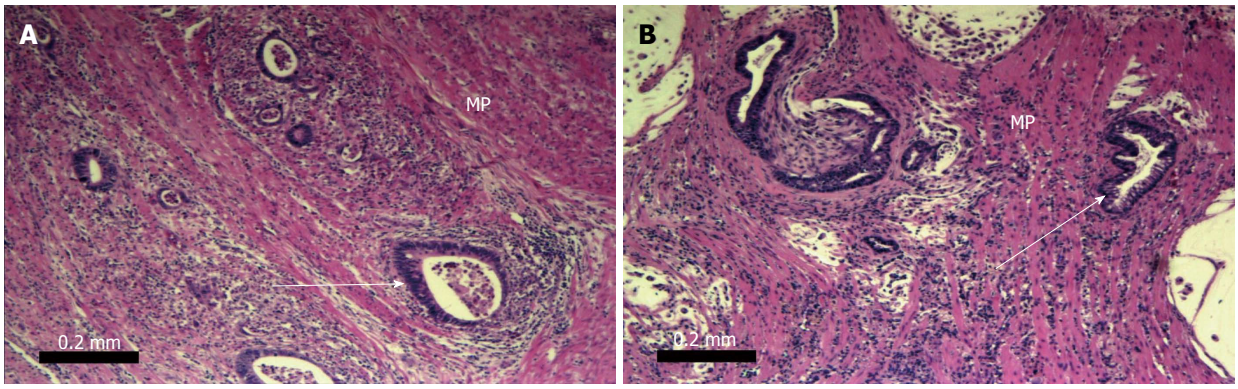

Figure 11 Invasion of the muscularis propria by adenocarcinoma stage T2, human (A) and mouse (B). Malignant glands (long arrows) can be seen invading the muscularis propria (MP). The pale areas within the stroma in B are mucin pools. Necrotic material is seen within the lumen of malignant glands in A and B. Images obtained with 10× objective lens.

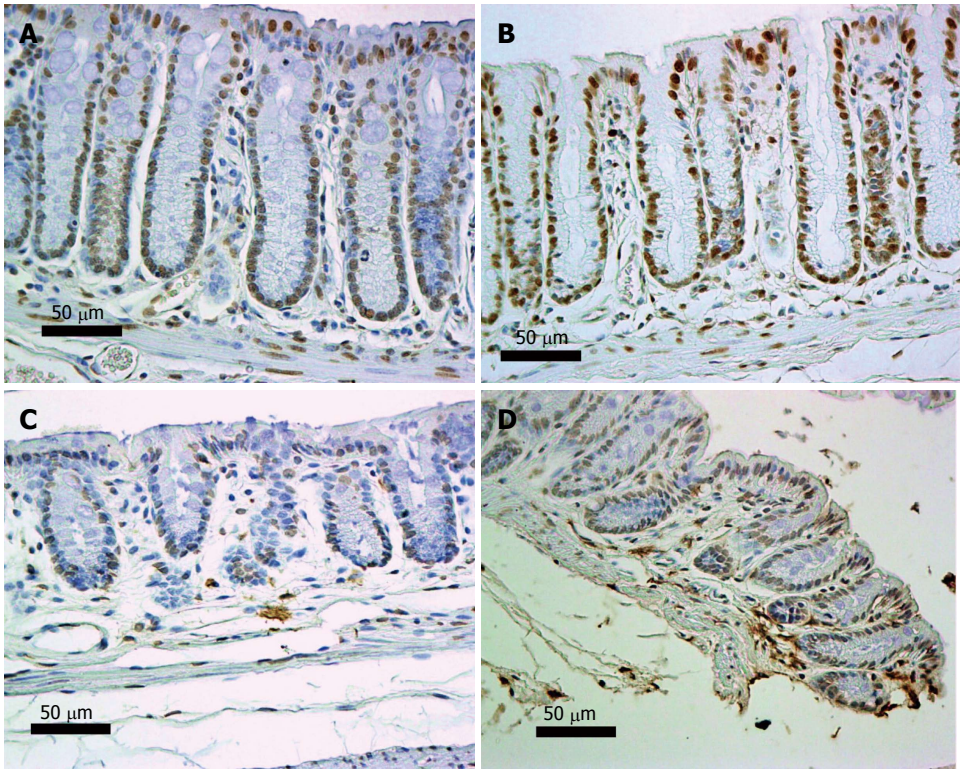

Figure 12 Colonic epithelia from mice fed diet + deoxycholic acid (A, B) or mice fed control diet (C, D) immunostained (brown) for 8-OH-dG, counter-stained with hematoxylin. Images obtained with 40× objective lens.

and for 56% of these mice the neoplasia had progressed to adenocarcinoma. There had been 12 mice fed diet + DOC + CGA, of which 64% developed colonic neoplasia, and for 18% of these mice the neoplasia had progressed to adenocarcinoma, so that CGA was somewhat protective against colonic neoplasia and adenocarcinoma

8-OH-dG in progression to colon cancer

As reviewed by Scott *et al*[14], the DNA damage 8-OH-dG is carcinogenic. Six mice, on their diets for 8 mo, were terminated and their colons removed for evaluation of nuclear 8-OH-dG (Figure 12). Three of these mice were on the standard diet and three had been fed diet + DOC. Colonic tissue sections from each mouse were placed on slides and immunostained for 8-OH-dG. Figure 12 shows tissues from 2 mice fed diet + DOC (Figure 12A and B) and 2 mice fed control diet (Figure 12C and D). Brown stain indicates 8-OH-dG and blue is hematoxylin stain for the chromatin in the nucleus. The level of 8-OH-dG was graded in the nuclei of the colonic crypt cells by IHC on a scale of 0-4. The nuclei of mice fed diet + DOC were largely at levels 3 to 4 (Figure 12A and B) while for mice fed diet alone were largely at levels 0 to 2 (Figure 12C and D). The images in Figure 12 were each uniform-

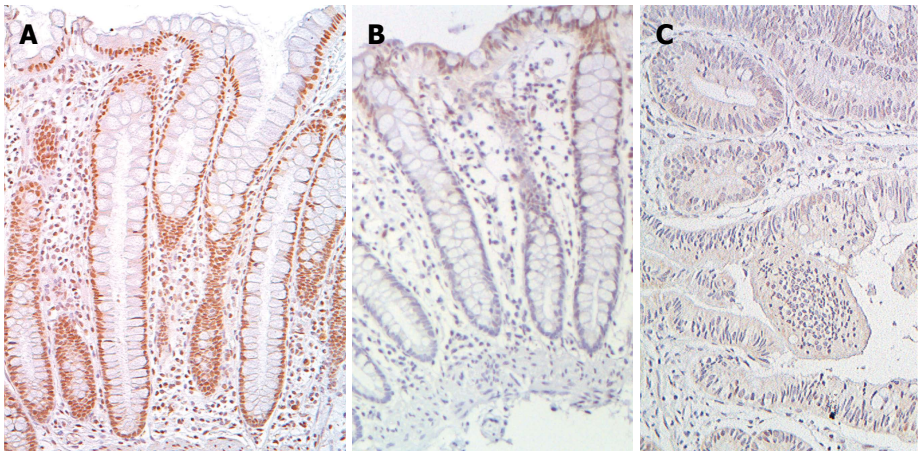

Figure 13 Human colonic mucosa immunostained (reddish brown) for excision repair cross-complementation group 1 with blue hematoxylin counter stain for chromatin. A: From patient without colonic neoplasia; B: From tissue near a colon cancer; C: From cancer tissue. Images with 40× objective. Scale shows 50 μm.

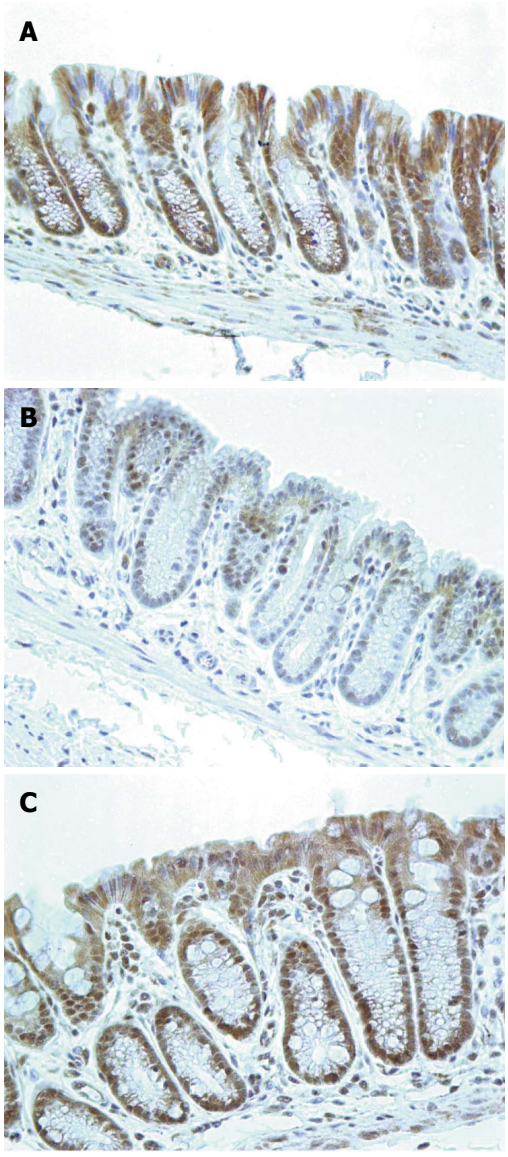

Figure 14 Mouse colonic epithelia with immunohistochemistry for excision repair cross-complementation group 1 (brown) and hematoxylin (blue) for chromatin. Mice fed diets: A: Control; B: Diet + deoxycholic acid (DOC); C: Diet + DOC + chlorogenic acid. Images obtained with 40× objective lens. Scale shows 50 μm.

ly enhanced in Paint Shop Pro 5 by increasing "shadow" to 35 and "saturation" to 35 to allow enhancement of the brown and blue colors for greater clarity in evaluating the immunohistochemical staining.

ERCC1 deficiency in progression to colon cancer

We recently reported that expression of DNA repair gene *ERCC1* was generally deficient in the histologically normal tissue surrounding human colon cancers (field defects susceptible to carcinogenesis) and in colon cancers themselves[11,12]. Figure 13 shows examples of IHC staining for ERCC1 of human colonic epithelia obtained during these previous studies. As shown in these images, the nuclei of cells in the colonic crypts of a patient without colonic neoplasm (Figure 13A) have high expression of ERCC1. However, in the crypts near a colon cancer (within 10 cm of a cancer in this example) (Figure 13B), cells in the lower parts of crypts (in the stem cell and proliferative regions) are usually deficient for ERCC1 while cells in the upper parts of the crypts and along the colonic lumen have restored ERCC1 expression. Within the area of a colon cancer (Figure 13C), ERCC1 is largely absent from the nuclei. The images in Figure 13 were each uniformly enhanced as described for Figure 12.

Nine mice, on their diets for 8 mo, were terminated and their colons removed for evaluation of expression of ERCC1. Three of these mice were on the standard diet, three had been fed diet + DOC and three had been fed diet + DOC + CGA. Colonic tissue sections from these mice were immunostained for ERCC1. Figure 14 shows typical colonic epithelial tissues from a mouse fed control diet (Figure 14A), a mouse fed diet + DOC (Figure 14B), and a mouse fed diet + DOC + CGA (Figure 14C). The colonic crypt cells of mice fed the control diet for 8 mo have high expression of ERCC1 (Figure 14A). For mice fed diet + DOC for 8 mo, cells in the lower parts of crypts are deficient for ERCC1 while the upper parts of the crypts usually have restored ERCC1 expression (Figure 14B). The cells of mouse colonic crypts of mice fed diet + DOC + CGA have high nuclear expression of ERCC1 (Figure 14C). The images in Figure 14 were each

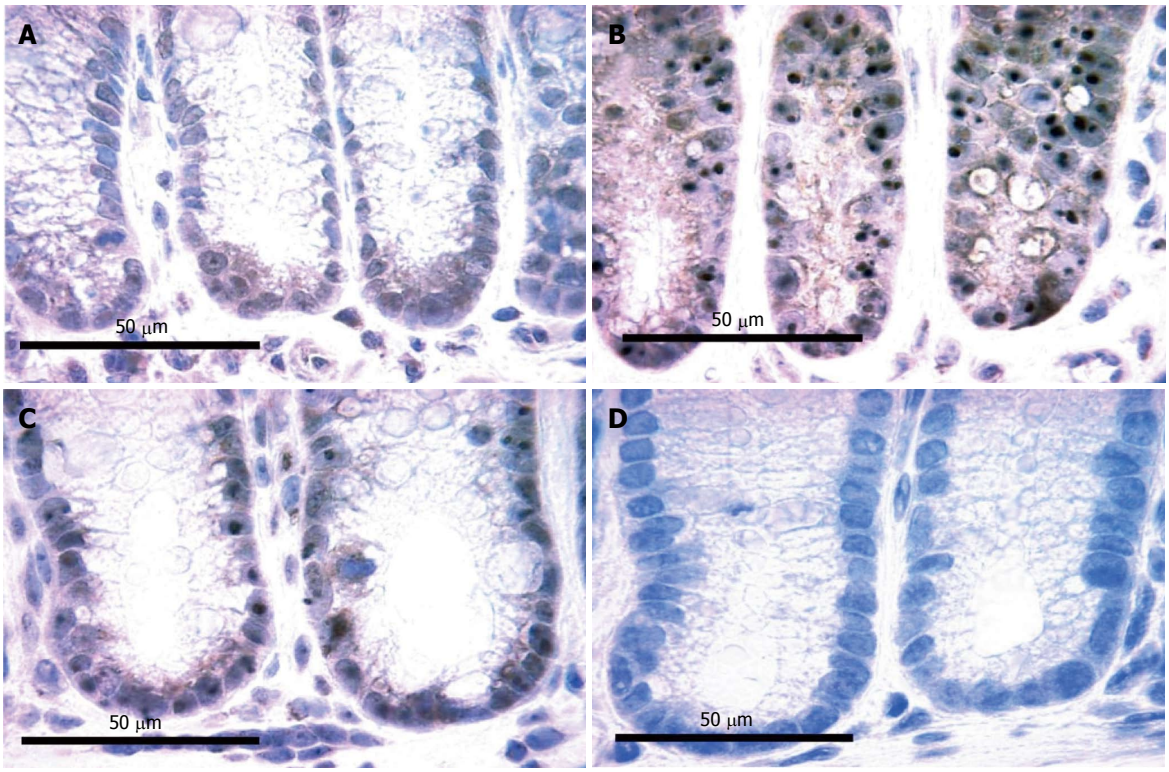

Figure 15 Immunohistochemistry of mouse colons for beclin-1. Mice fed diets: A: Control; B: Diet + deoxycholic acid (DOC); C: Diet + DOC + chlorogenic acid; D: Negative control without primary antibody (blue hematoxylin stain for nuclei). Images taken with 40× objective lens.

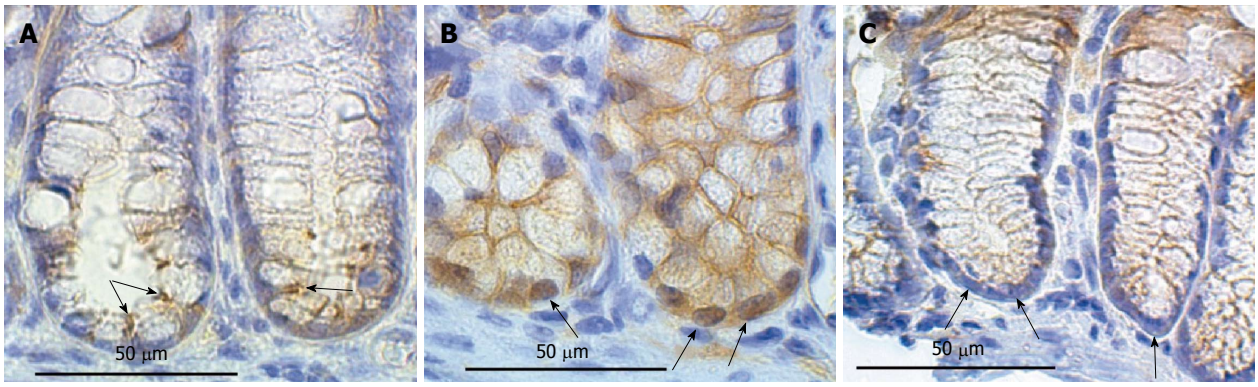

Figure 16 Lower regions of mouse colonic crypts immunostained for beta-catenin. Mice fed diets: A: Control; B: Diet + deoxycholic acid (DOC); C: Diet + DOC + chlorogenic acid (CGA). In A (control diet), in the stem cell region (lowest cells in the crypts), cells have beta-catenin expression localized to their membrane regions as shown by arrows. In B (diet + DOC), the stem cell region shows substantial nuclear localization of beta-catenin (arrows). In C (diet + DOC + CGA), stem cell region nuclei are largely deficient in beta-catenin, and the cytoplasm has low levels of beta-catenin, similar to the levels in mice fed the control diet alone. Images taken with 40× objective lens.

uniformly enhanced as described for Figure 12.

The mice fed the control diet had expression of ERCC1 (Figure 14A) that matched human ERCC1 expression for humans without colonic neoplasia (Figure 13A). The mice fed diet + DOC (and generally progressing to colonic neoplasia) had ERCC1 expression (Figure 14B) that matched human ERCC1 expression in a field defect giving rise to a cancer (Figure 13B). The mice fed diet + DOC + CGA, which had substantially fewer cancers[9], also had a level of ERCC1 expression (Figure 14C) that was similar to that of mice fed the control diet (Figure 14A).

Increased beclin-1 in progression to cancer

Beclin-1 is a central player in autophagy. The modulation of macroautophagy is now recognized as one of the hallmarks of human cancer cells[15]. Figure 15 shows colonic epithelium of mice immunostained for beclin-1, where the mice in Figure 15A-C were fed different diets for 8 mo. The level of beclin-1 was graded in the colonic crypt cells by IHC on a scale of 0-4. In the colonic epithelium of mice fed the control diet for 8 mo (Figure 15A) beclin-1 staining was at level 1. For mice fed diet + DOC (Figure 15B), expression was at level 4, and for mice fed diet + DOC + CGA expression was at level

Figure 17 Two mice, raised in the same pan, had different weights after 10 mo on their diet. The heavier mouse and the lighter mouse both appeared to be healthy and active.

3. For mouse colonic epithelium stained without the primary antibody (Figure 15D), staining was at level 0. These images were not enhanced.

Increased nuclear beta-catenin in the stem cell region in progression to cancer

The images in Figure 16 show the lower regions of mouse colonic crypts (including the stem cell regions) of mice that had been placed on three different diets for 8 mo - control diet, diet + DOC or diet + DOC + CGA. The colonic stem cell region showed only membrane expression of beta-catenin in samples of colonic epithelial tissue from all three of "control diet"-fed mice that were assessed here (one example is shown in the figure). The colonic stem cell region showed high nuclear expression of beta-catenin in samples of colonic epithelial tissue from all three of these diet + DOC fed mice that were assessed here (one example is shown in the figure). The colonic stem cell regions showed very low levels of beta-catenin in samples of colonic epithelial tissue from all three of the mice fed diet + DOC + CGA that were assessed here (one example is shown in the figure). The images in Figure 16 were each uniformly enhanced as described for Figure 12.

Weight distributions

The final weights of mice fed the control diet for 10 mo were quite varied, with the lowest weight being 25.2 g and the highest being 63.1 g. The mouse with the median weight was at 41.3 g. The distribution of weights for mice fed diet + DOC varied from 18.7 to 78.6 g, with a median weight of 35.5 g. Each mouse was weighed weekly, and no weight loss was detected for any of the mice during their 10 mo on each diet. Mice with relatively low weights at the end of 10 mo on their diets merely gained weight more slowly than heavier mice.

Each mouse, without respect to weight, appeared to be healthy and active (Figure 17). The variation in mouse weights, like the variation in colors of these mice (Figure 1), was likely due to the variation in their genetic constitutions. As pointed out in the Materials and Methods, the mice were the second generation (F2) of a cross between two well established, inbred, wild-type strains: C57BL/6J and 129S1/SvlmJ. The phenotypes of these F2 wild-type mice is expected to be varied, since the contribution of the two parental wild-type strains will be different in each F2 offspring. The varied weights of these mice may mimic the weight variations in the general human population.

A SKEW calculation on all the data had a value of 0.0896 indicating it was approximately symmetrically distributed. A *t* test was then applied to determine if there were significant differences between the weights of the control-fed and the diet + DOC-fed mice, using the assumption of unequal variances (since the variances were different). The two-tailed *t* test, which indicates if the differences between the two populations are larger or smaller than each other, gave a *P*-value of 0.159, indicating that there is no significant difference between the two populations in distributions of weight. An ANOVA analysis using the same datasets also gave a *P*-value of 0.159. Thus distributions of weights were similar and there was no significant difference between the weight distributions for the two types of diets. There was also no systematic association of type of tumor development with weight of the mice fed diet + DOC. A Pearson correlation analysis determined the weight of the mice fed a DOC-supplemented diet was not correlated to the number of colonic tumors found ($P = 0.78$).

DISCUSSION

Similarity of DOC in diet + DOC mouse colons to that of humans on a high fat diet

For humans on a non-controlled omnivorous diet in London England, the level of DOC in the feces averaged 3.2 mg/g dry weight[16]. A high fat human diet in the United States doubles the colonic DOC concentrations[17] and would subject people to colonic exposure to DOC at an average value in their feces of about 2 × 3.2 mg/g = 6.4 mg/g dry weight. Addition of 0.2% DOC for 6 mo to the diet of 18 wild-type male mice produced mouse feces with 4.6 mg DOC/g dry weight (comparable to the 6.4 mg/g dry weight for humans on a high fat diet). Mice on a control diet for 6 mo, on the other hand, had feces with less than a tenth the level of fecal DOC, having 0.3 mg DOC/g dry weight. Among the 18 mice fed diet + DOC, 17 developed colonic tumors in our previous study[9], including 10 mice with colon cancers. In our present study, using female mice instead of male mice, we confirmed a high frequency of colon cancer (10 of 22 mice) with mice fed diet + DOC.

Parallel histology of mouse model colon tumors and human colonic tumors

Histopathologic evaluation constitutes the gold standard for determining progression of colonic epithelium to colon cancer, to which other methods are compared[10]. Using histopathologic evaluation, we showed that mice fed diet + DOC progress to colon cancer in a manner closely

similar to such progression in humans.

We found that tumors in these diet + DOC fed mice mimic each of the histopathologic features of progression to colon cancer in humans that we tested. The features illustrated in Figures 6-11 include tubular adenomas, tubular adenomas with high grade dysplasia, sessile serrated adenomas, adenocarcinomas of category T1 (cancers that have invaded through the muscularis mucosa and extended into the submucosa), and adenocarcinomas of category T2 (cancers that have invaded through the submucosa and into the muscularis propria). As in the great majority of humans progressing to colon cancer, no tumors were found in the small intestines of these DOC-fed mice.

Locations of tumors in our mouse model and in humans

All of the tumors found in our previous study with male mice[9] were in the proximal colons of the mice. In our current study with female mice, the majority of tumors were in the proximal colon, with 44 of the 57 tumors or 77% of tumors being in the proximal colon. This is somewhat different from tumors in the human colon where tumors are found to be more nearly equally distributed between the proximal and distal regions of the colon. However, the level of DOC in the different regions of the human colon depends on two factors, while it was primarily dependent on only one factor in the mice fed diet + DOC. The first factor in humans is the continuous deconjugation and dehydroxylation (by bacteria) of the cholic acid entering the colon from the small intestine. This bacterial action generates newly formed DOC throughout the length of the colon[18]. The second factor in humans is the high level of absorption (about 50% overall) of DOC as it passes along all the regions of the colon[19]. In humans, the level of DOC would be about the same throughout the colon. In our mouse model, on the other hand, the level of DOC in mice fed diet + DOC starts off high in the proximal region of the colon. In contrast to humans, conversion of cholic acid to DOC is likely relatively insignificant for these mice since about 90% of the DOC in the colons of the mice fed diet + DOC comes from the added DOC in the diet rather than from conversion of cholic acid to DOC in the colon. Presumably, there is similar absorption of DOC from all regions of the colon in mice, as occurs in humans. Thus, there should be higher levels of DOC in the proximal regions of the colons of the mice compared to that in their distal regions. In our mice, much of the DOC would be absorbed as it travels down the length of the mouse colon. If tumors are caused by interaction of relatively high levels of DOC with colonic epithelial cells, then it is likely that, in our system, the majority of tumors would occur in the proximal colons of the mice, while in humans, with a more even distribution of DOC along the colon, tumors would occur in both the proximal and distal regions of the colon.

Tumors and colon cancers in mice occurred at an earlier age than normally found in humans. However, as reviewed by Cortopassi *et al*[20], multiple studies show that mice have about a 5.9-fold lower level of DNA repair than humans. A model proposed by these authors suggests that the earlier occurrence of colon cancer in mice fed diet + DOC, compared to humans, could be due to the DNA damaging nature of DOC and the lower DNA repair rate in mice.

Field defects in progression to cancer

Colon cancers are known to arise within a "field defect," an area of the colon predisposed to progression to cancer[21]. As pointed out by Rubin[22], field defects are of crucial importance in progression to cancer. Multiple tumors in a localized area during progression to colon cancer indicate a field defect.

Macroscopically, we found multiple colonic tumors in the same colonic area, indicating that colonic tumors in both mice and humans often occur within a field defect. We previously reported, by immunhistochemical evaluation, that the colonic mucosa surrounding human colon cancers has biomarker alterations indicative of a field defect as well[11]. We can speculate that some of the mutant or epigenetically altered cells are produced due to an early deficiency in ERCC1 (and possibly to deficiencies in other un-evaluated DNA repair proteins). Such cells would be genetically unstable and could acquire a growth advantage (*e.g.,* apoptosis resistance) due to further mutations and/or epimutations. We have shown that colonic epithelial cells grown in culture and repeatedly exposed to increasing concentrations of DOC underwent natural selection to develop resistance to apoptosis[23]. These apoptosis-resistant cells were altered in expression in 839 out of 5000 genes assessed by cDNA assay[23] and in 91 of 454 proteins detected by a proteomic analysis[24]. Cells with a growth advantage, upon proliferation, may form a defective field, which, with further mutation and epigenetic alteration due to bile acids, and further selection, could give rise to tumors, and eventually, to a colon cancer.

Oxidative DNA damage, the antioxidant CGA, and DNA repair in colon cancer

As reviewed by Bernstein *et al*[25] exposure of colon cells to high physiologic concentrations of DOC increases formation of reactive oxygen species (ROS), increases DNA damage, and causes apoptosis. A particularly important oxidative damage to DNA is 8-OH-dG, considered to play a central role in carcinogenesis[14]. A central enzyme in repair of oxidative damage to DNA is ERCC1[26]. In our present study 8-OH-dG is substantially increased and protein expression of ERCC1 is substantially decreased in the colonic epithelium of mice fed diet + DOC and progressing to colon cancer.

Chlorogenic acid (CGA) is an ester formed between caffeic acid and quinic acid, and is widely available in many food products, especially in coffee, blueberries and eggplant[27,28] and can even be purchased as diet supplement capsules containing 50% CGA. CGA is an excellent natural scavenger of free radicals because the one-elec-

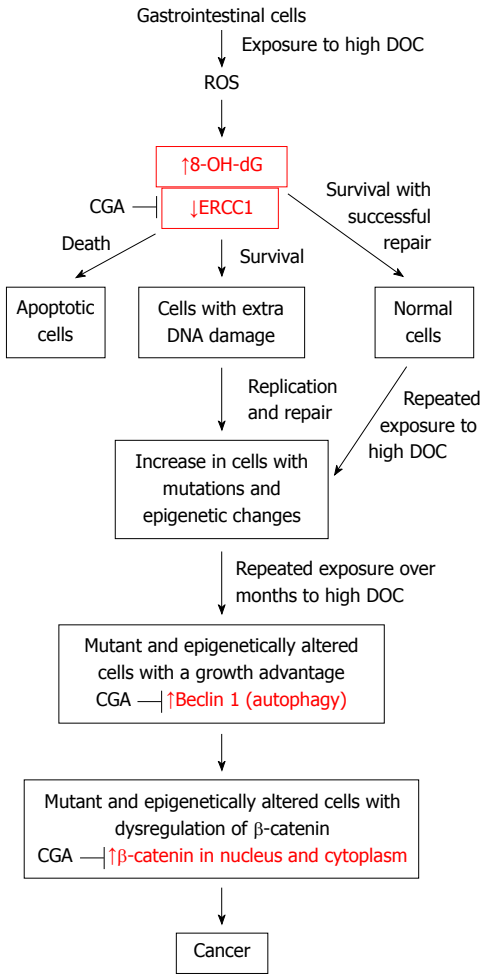

Figure 18 Likely path of progression to colon cancer in mice and humans, indicating key roles of the molecular markers evaluated here and the points of effects of chlorogenic acid in mice. CGA: Chlorogenic acid; DOC: Deoxycholic acid; ERCC1: Excision repair cross-complementation group 1.

tron oxidation product of CGA formed by the reaction with free radicals is rapidly broken down to products that cannot generate further free radicals[29].

We previously tested 19 antioxidants to evaluate their effect on expression of DNA repair proteins[30]. Only chlorogenic acid (CGA) and its metabolic derivatives increased expression of two DNA repair enzymes in that study. In our previous report on our new diet-related mouse model of colon cancer[9], CGA, fed to mice at a level equivalent to three cups of coffee a day for humans, substantially reduced the incidence of colon cancer for mice fed diet + DOC. Here, CGA in the diet largely prevented the reduction in protein expression of DNA repair protein ERCC1, central to repair of oxidative damage to DNA, that otherwise occurs with feeding mice diet + DOC.

Beclin-1 and autophagy

Beclin-1 is a central player in autophagy. The modulation of autophagy is now recognized as one of the hallmarks of human cancer cells. Accumulating evidence indicates that autophagy plays a role in the various stages of tumorigenesis. Depending on the type of cancer and the context, macroautophagy can be a tumor suppressor or it can help cancer cells to overcome metabolic stress and advance[15]. In particular, beclin-1 appears to be a central player in the mechanisms that control the level of p53. In addition, beclin-1 activates the autophagic pathway and this contributes to apoptosis resistance, which might have a role in carcinogenesis[31]. In mouse colonic epithelial tissues beclin-1 was increased in mice fed diet + DOC (Figure 15B), but this increase was reduced in mice fed diet + DOC + CGA (Figure 15C).

Beta-catenin in progression to cancer

Four major signaling pathways are frequently altered in the later stages of progression to sporadic human colon cancer, and three other pathways have also been identified. The most frequent pathways are Wnt/beta-catenin, TGF-beta receptor, Notch, and Hedgehog, while the other pathways are the EGFR, RAS/RAF/MAPK cascade and PI3K/Akt[32]. No one pathway is altered in all sporadic colon cancers. However, beta-catenin nuclear accumulation is found in 40% to 80% of primary human colon cancers[33,34] and in 67% of sessile serrated adenomas progressing towards human colon cancer[35]. We assessed beta-catenin and found that it is translocated into the nucleus of cells in the stem cell region of mouse colonic crypts in mice fed diet + DOC, but this translocation is reduced if CGA is also added to the diet (indicated in Figure 16).

Difficulties with previous rodent models of colon cancer

Rosenberg *et al*[36], in a 2009 review of then-current mouse models of colonic carcinogenesis, noted that they lack an invasive phenotype. Corpet *et al*[37] noted in 2005 that most then-current rodent models of colonic carcinogenesis did not share several significant genetic events and histopathological features of human colon cancers.

In the New Western diet (NWD)[38] mouse model of colon cancer (based on a diet deficient in calcium, vitamin D_3, fiber, methionine and choline, plus increased corn oil) mice developed the same frequency (4 out of 15 mice) of small intestinal tumors as colon tumors after 2 years on the diet. This is unlike intestinal cancers in humans where only 6% as many small intestinal cancers develop compared to the frequency of colon cancers[39]. In addition, no mice solely on the NWD developed fully invasive colonic adenocarcinomas[38].

Pathway of progression to colon cancer

A likely pathway for progression to colon cancer is shown in Figure 18. This figure indicates presumed major steps in progression to colon cancer. The key roles of the molecular markers we evaluated in our diet-related mouse model of colon cancer are shown in red. The effect of CGA on these markers is also indicated by arrows.

Bile acids, especially DOC, cause increases in DNA damaging ROS in colon cells[40-43]. DOC-induced ROS

are shown in Figure 18 as an early step in our diet-related pathway to colon cancer.

A major type of DNA damage caused by ROS is 8-OH-dG[44]. 8-OH-dG is mutagenic[45], and an initiator of carcinogenesis[14]. Thus, increased 8-OH-dG, as found by us in the epithelium of mice fed diet + DOC, is shown in Figure 18 as a key step in progression to colon cancer.

DNA damage appears to be a primary underlying cause of cancer[46]. Cells that retain unrepaired DNA damage, upon replication, may give rise to daughter cells with increased mutations by translesion synthesis[47,48]. Inaccurate or incomplete repair of DNA damages may also give rise to mutations or epigenetic alterations[49,50]. Such increased mutations and epigenetic alterations likely underlie progression to cancer, as indicated in Figure 18.

Deficiencies in DNA repair genes and genomic instability

In sporadic cancers, a deficiency in DNA repair may sometimes occur due to a mutation in a DNA repair gene. However, much more frequently, reduced or absent expression of DNA repair genes occurs due to epigenetic alterations that reduce or silence gene expression. For example, for 113 colorectal cancers examined in sequence, only four had a missense mutation in the DNA repair gene *MGMT*, while the majority had reduced MGMT protein expression due to methylation of the *MGMT* promoter region (an epigenetic alteration)[51]. Similarly, out of 119 cases of mismatch repair-deficient colorectal cancers that lacked DNA repair gene *PMS2* expression, PMS2 protein was deficient in 6 due to mutations in the *PMS2* gene, while in 103 cases PMS2 protein expression was deficient because its pairing partner the MLH1 protein was epigenetically repressed due to promoter methylation (PMS2 protein is unstable in the absence of MLH1 protein)[52]. In the other 10 cases, loss of PMS2 protein expression was likely due to epigenetic over-expression of the microRNA, miR-155, which down-regulates MLH1 protein expression[53]. Epigenetic deficiencies in expression of DNA repair proteins are virtually always present in colon cancers[46]. Epigenetically caused DNA repair protein deficiencies and the frequencies with which they are reported in colon cancers are MSH2 (13%), MLH1 (2%-65%), WRN (38%), MGMT (46%-90%), XPF (55%), PMS2 (88%) and ERCC1 (100%)[46]. ERCC1 protein deficiency was observed in all of the 47 human colon cancers evaluated[11] and thus ERCC1 deficiency appears to be one of the most prevalent DNA repair deficiencies in progression to colon cancer in humans. ERCC1 protein was also found to be deficient in histologically normal colonic epithelial tissues in mice fed diet + DOC and progressing to colon cancer (Figure 14).

A major characteristic of cancer is the presence of genomic instability (a mutator phenotype)[54]. This may be due to deficiency of a human DNA repair enzyme, such as ERCC1[11,46]. The average colon cancer has about 60 to 70 protein altering mutations of which about 3 or 4 may be "driver" mutations[55]. However, the protein coding part of the genome is only about 1.5% of the entire genome[56]. There are also about 20000 to 80000 mutations in the entire genome of various cancers[57,58]. This compares to the very low mutation frequency of about 70 new mutations in the entire genome between generations (parent to child) in humans[59,60]. The very high mutation frequency in cancer cells may be due to the frequent epigenetic deficiencies in DNA repair genes that likely occur early in progression to cancer. This is illustrated near the top of Figure 18. ERCC1 deficiency may have a major role in genomic instability in colon cancers. In our present study, mice progressing to colon cancer are deficient in protein expression of ERCC1 in the stem cell regions of colonic crypts.

The diet-related mouse model of colon cancer described here appears to be the closest model to human development of colon cancer that is currently available. It is based on elevated colonic levels of the natural endogenous bile acid DOC, long thought (from epidemiological evidence) to be important in initiation and progression to colon cancer[6,7]. It closely parallels human progression to colon cancer, both by the gold standard of histopathology and by the molecular markers tested. This mouse model may be uniquely useful in experiments involving the prevention or treatment of colon cancer.

COMMENTS

Background

Colon cancer is the second most frequent cause of cancer mortality among men and women combined, in both more developed and less developed areas of the world. Diet appears to be the major factor affecting frequency of colon cancer. Up to now, however, there has not been an established diet-related rodent model that closely parallels human progression to colon cancer. Such a model is needed to have an effective basis for experiments exploring the prevention or treatment of colon cancer.

Research frontiers

Bile acids delivered to the colon in response to a high fat diet have long been hypothesized to have a key role in development of colon cancer. In support of this hypothesis, it was recently found that the concentration of the bile acid deoxycholate in the feces of native Africans is only 1/5th as high as in African Americans, and the frequency of colorectal cancer in native Africans is less than 1/72nd the frequency of colorectal cancer in African Americans. An important area of research is to determine the molecular changes and neoplastic consequences caused by increased deoxycholate in the colon.

Innovations and breakthroughs

The study of experimental colon carcinogenesis in rodents has a long history, dating back about 70 years to an experiment of adding methylcholanthrene to the food of mice. Most studies were done with potent chemical carcinogens, which would not likely cause the same types of DNA damages that are caused by natural dietary factors. More recently, studies were also done with transgenic, knockout and knockin genetic models. In addition, a mouse model of colon cancer (based on a diet deficient in calcium, vitamin D_3, fiber, methionine and choline, plus increased corn oil) was devised. A notable disadvantage of these models was that induced tumors generally lacked an invasive and metastatic phenotype, and for many models, small intestinal neoplasias were often as frequent (or more frequent) than colon cancers, unlike the situation in humans. In addition, mutational alterations frequently present in human colon cancers were often not present in artificial rodent models of colon cancer. Thus, the finding that the natural endogenous bile acid deoxycholate actually caused colon cancer in a mouse model is an important contribution. Authors consider that this model should produce the typical types of DNA damages produced in humans by high physiologic levels of bile acids. Also, this model only produces cancers

in the colon, the location of almost all human intestinal cancers. Authors now show that the cancers produced are invasive and have morphological features and molecular markers consistent with those found in human progression to colon cancer.

Applications

The results of the present study indicate that this diet-related mouse model of colon cancer (with human physiologic levels of deoxycholate) will provide a more effective basis for experiments exploring the prevention or treatment of colon cancer than has previously been available.

Terminology

Human physiologic levels of deoxycholate are levels of deoxycholate found in humans eating a diet high in milk fat (sour cream, butter) and beef fat, or high in corn oil. Cancer mortality is the frequency of deaths due to a particular form of cancer.

Peer review

This study analyzes a novel diet-related model of colon cancer that parallels human progression to colon cancer, using both histomorphological criteria and molecular biomarkers. It also shows the ameliorating effects of dietary chlorogenic acid (a common component of blueberries, eggplant and apples) on molecular biomarkers of progression to colon cancer. This study is, undoubtedly, highly relevant for future research in human colonic cancer.

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ORIGINAL ARTICLE

Growth inhibition of colon cancer cells by compounds affecting AMPK activity

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Abstract

AIM: To determine if other molecules reported to modulate AMP-dependent protein kinase (AMPK) activity would have effects resembling those of metformin and phenformin on colon cancer cell proliferation and metabolism.

METHODS: Studies were performed with four human colon cancer cell lines, Caco-2, HCT116, HT29 and SW1116. The compounds that were studied included A-769662, 5-aminoimidazole-4-carboxamide-1-ribofuranoside, butyrate, (-)-epigallocatechin gallate (EGCG), KU-55933, quercetin, resveratrol and salicylates. The parameters that were measured were cell proliferation and viability, glucose uptake, lactate production and acidification of the incubation medium.

RESULTS: Investigations with several molecules that have been reported to be associated with AMPK activation (A-769662, 5-aminoimidazole-4-carboxamide-1-b-D-ribofuranoside, EGCG, KU-55933, quercetin, resveratrol and salicylates) or AMPK inhibition (compound C) failed to reveal increased medium acidification and increased glucose uptake in colon cancer cells as previously established with metformin and phenformin. The only exception was 5-aminosalicylic acid with which there were apparently lower glucose levels in the medium after incubation for 72 h. Further study in the absence of cells revealed that the effect was an artifact due to inhibition of the enzyme-linked glucose assay. The compounds were studied at concentrations that inhibited cell proliferation.

CONCLUSION: It was concluded that treatment with several agents that can affect AMPK activity resulted in the inhibition of the proliferation of colon cancer cells under conditions in which glucose metabolism is not enhanced, in contrast to the effect of biguanides.

Key words: Colon cancer cells; Proliferation; AMP-dependent protein kinase; Glucose metabolism

Core tip: Treatment with several agents that can affect AMP-dependent protein kinase activity resulted in the inhibition of the proliferation of colon cancer cells under conditions in which glucose metabolism is not enhanced, in contrast to the effect of biguanides.

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INTRODUCTION

In previous publications we reported that the biguanides, metformin and phenformin, inhibited proliferation of

colon cancer cells under conditions in which glucose uptake was increased and there was increased glycolysis as judged by acidification of the incubation medium[1,2]. This is an unusual combination of effects and raises the question of whether other molecules might have similar action. Although the biguanides have a long history in the treatment of Type II diabetes there has been uncertainty regarding their mechanism of action[3,4]. Interest in mechanisms has been further stimulated by observations that metformin may exert a cancer chemopreventive effect and this has led to ongoing clinical trials against different types of cancer[5,6]. The most commonly suggested mechanisms for the action of biguanides include a stimulation of AMP-dependent protein kinase (AMPK) and inhibition of complex I in the mitochondrial electron transport chain. The hypothesis to be tested in the present work was that other molecules reported to modulate AMPK activity would have effects on colon cancer cell proliferation and metabolism resembling those of biguanides. We chose to examine the action of a variety of compounds that have been reported to activate or inhibit AMPK. Activators included A-769662[7], 5-aminoimidazole-4-carboxamide-1-b-D-ribofuranoside (AICAR)[8], (-)-epigallocatechin gallate (EGCG)[9], KU-55933[10], quercetin[11], resveratrol[12] and salicylates[13]. The most widely studied inhibitor of AMPK is compound C and that compound has been shown to affect proliferation of colon cancer cells[1]. Butyrate has been most commonly considered as an inhibitor of histone deacetylase activity but activation of AMPK by butyrate has been reported. In a previous study we observed that the induction of alkaline phosphatase by butyrate in colon cancer cells was not significantly affected by coincubation with A-769662[1]. However, in the present work some additive effects on metabolism and cell proliferation have been seen after coincubation of butyrate and A-76992 with colon cancer cells.

MATERIALS AND METHODS

Cells and determination of cell proliferation

SW1116, HCT116, HT29, and Caco-2 human colon cancer cells were obtained from the American Type Culture Collection, Rockville, MD, United States, and were incubated at 37 ℃ in RPMI-1640 medium with 5% fetal calf serum. Of these cell lines, the HCT116 cells exhibited the most rapid proliferation, and the slowest growth was seen with the SW1116 cells. Cell proliferation was generally monitored by the increase in protein. In studies with 96-well plates, the procedure involved staining with sulforhodamine B essentially as described by Vichai *et al*[14]. Cells were routinely allowed to attach to tissue culture dishes or 96-well plates for 24 h before changing the medium. The cells were then incubated for a further 72 h before determining the impact of the compounds under study on medium pH, glucose concentration, and cell proliferation as judged by protein mass. Cell viability was monitored using the Presto Blue Viability Reagent from Life Technologies Corporation, Carlsbad, CA, United States.

Reagents

A-769662 was purchased from LC Laboratories, Woburn, MA, United States. AICAR, butyrate, (-)-epigallocatechin gallate, metformin, phenformin, quercetin, resveratrol, salicylic acid, acetylsalicylic acid, 4-aminosalicylic acid and 5-aminosalicylic acid were obtained from Sigma-Aldrich, St. Louis, MO, United States. KU-55933 was purchased from Selleck Chemical, Houston, TX, United States.

pH determination

pH determination with an electrode was found previously to correlate well with changes in the light absorbance at 560 nm reflecting changes in the pH indicator, phenol red, where a higher absorbance reflects a higher pH[1]. The latter method was found particularly convenient for work with 96 well plates and was used routinely in the present work.

Glucose assay

Glucose was assayed in the cell culture medium using GAGO-20 Kit from Sigma-Aldrich. This is a colorimetric procedure in which the oxidation of glucose is coupled with glucose oxidase and peroxidase to the oxidation of dianisidine.

Lactate assay

Lactate in the medium was determined using the assay kit obtained from Eton Bioscience, San Diego, CA, United States.

Statistical analysis

Data are presented as means and standard deviations. Statistical significance of the results was determined by a two-tailed Student's *t* test or by Dunnett's test for multiple comparisons using the Instat program from GraphPad Software, Inc., La Jolla, CA, United States. A probability of less than 5% was considered significant and differences compared to the control are shown.

RESULTS

The uptake of glucose by HCT116 colon cancer cells was inhibited by incubation with butyrate or A-769662 for 72 h. This is shown in Figure 1A where the final glucose concentrations in the medium are shown after an initial glucose concentration of 2 mg/mL. Decreased glucose uptake paralleled decreased acidification of the incubation medium (Figure 1B) and decreased lactate production (Figure 1C). The data in Figure 1D indicate inhibitory effects of butyrate and A-769662 on proliferation of HCT116 cells as judged by staining with sulforhodamine B. The data in Figure 2A-D show similar responses in HT29 cells to those seen with HCT116 cells. Measurement of metabolic activity in HT29 cells as reflected in

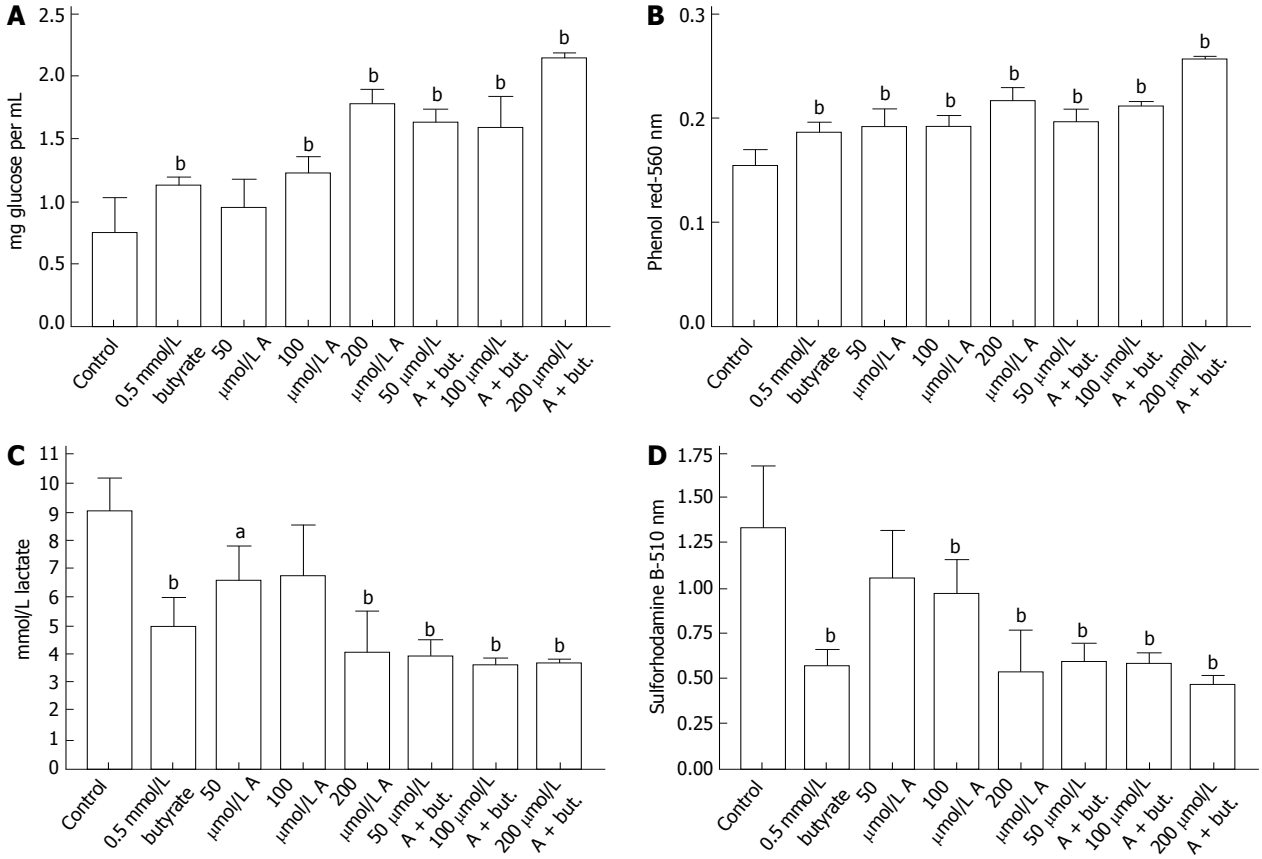
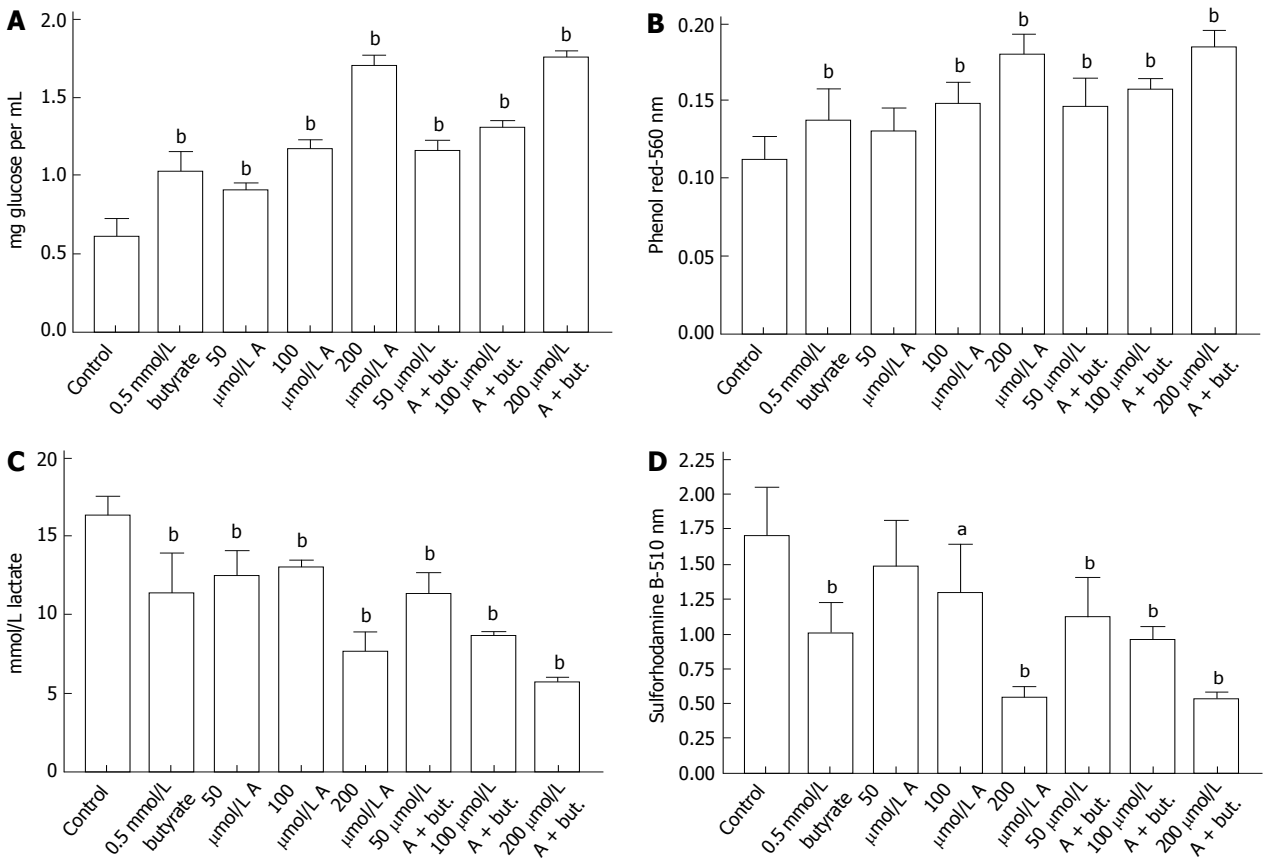


Figure 1 Effects of incubation of HCT116 WT cells for 72 h with butyrate. A-769662 (A) on glucose concentration of the incubation medium (A), medium pH (B), medium lactate concentration (C), and cell proliferation (D). $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control group.



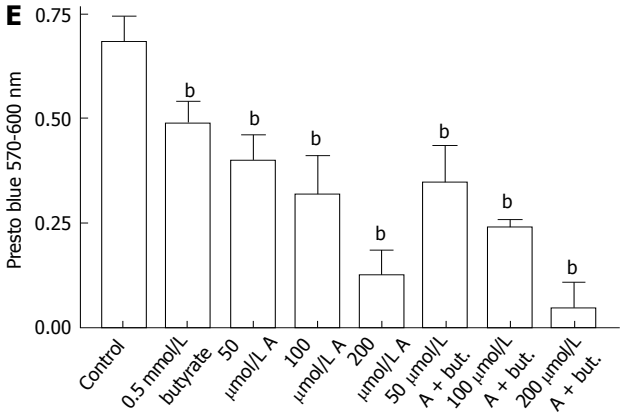


Figure 2 Effects of incubation of HT29 cells for 72 h with butyrate. A-769662 (A) on medium glucose concentration (A), medium pH (B), medium lactate concentration (C), cell proliferation (D) and reduction of Presto Blue (E). $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control group.

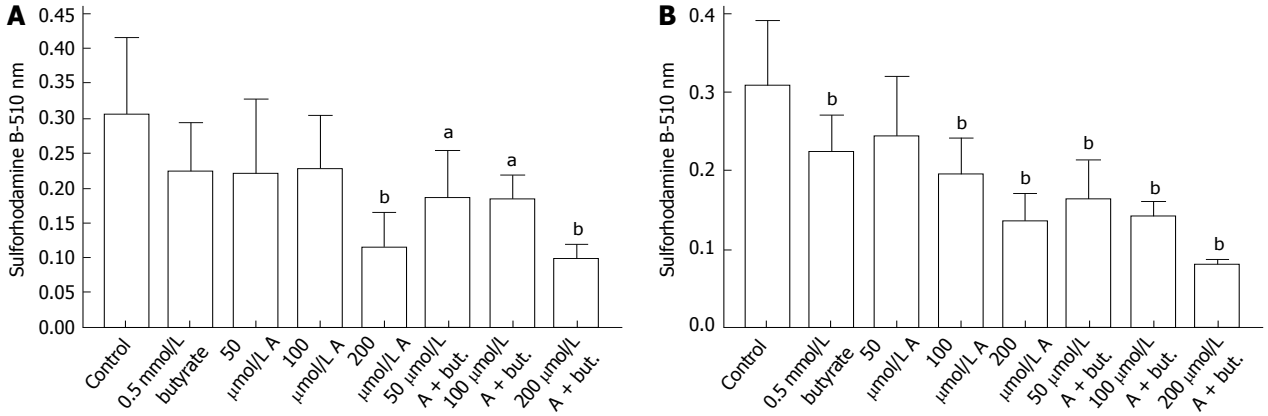


Figure 3 Effects of incubation of Caco-2 cells (A) and SW1116 cells (B) for 72 h with butyrate (but) and A-769662 (A) on cell proliferation. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control group.

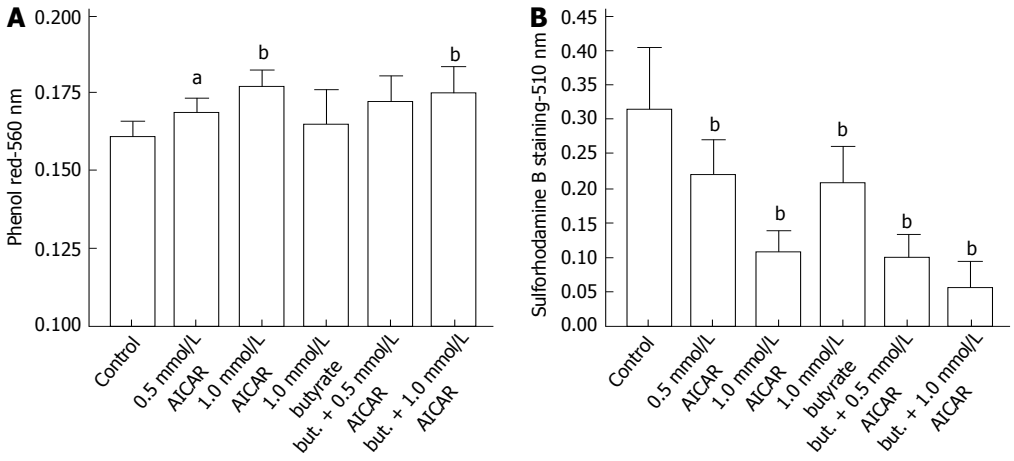


Figure 4 Effects of incubation of Caco-2 cells for 72 h with butyrate (but) and AICAR on medium acidification (A) and cell proliferation (B). $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control group.

the reduction of Presto Blue show a similar profile to that seen with sulforhodamine B staining and suggest some additive action when there is coincubation with butyrate and A-769662 (Figure 2E). Effects on metabolism in the more slowly growing Caco-2 and SW1116 cells were not as marked as in the more rapidly growing HT29 and HCT1116 cells but the results in Figure 3 show some degree of additive effect of butyrate and A76992 on the inhibition of cell proliferation.

Significant effects on glucose uptake were not seen when Caco-2 cells were incubated for 72 h with 0.5 and 1 mm AICAR but as shown in Figure 4A there were increases in medium pH suggesting less glycolysis and this was accompanied by decreased proliferation (Figure

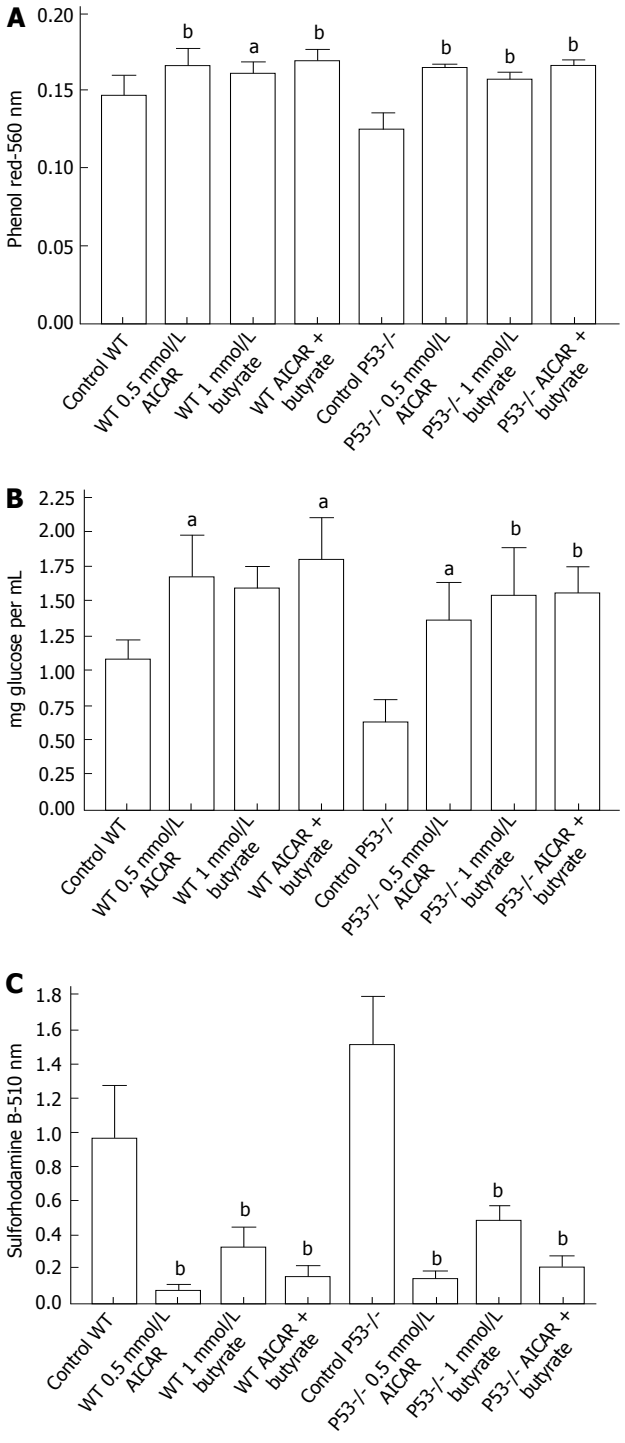


Figure 5 Effects of incubation of HCT116 wild type or p53 null cells. Effects of incubation of HCT116 wild type or p53 null cells for 72 h with butyrate and AICAR on acidification of the incubation medium (A), medium glucose concentration (B) and cell proliferation (C). $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control group.

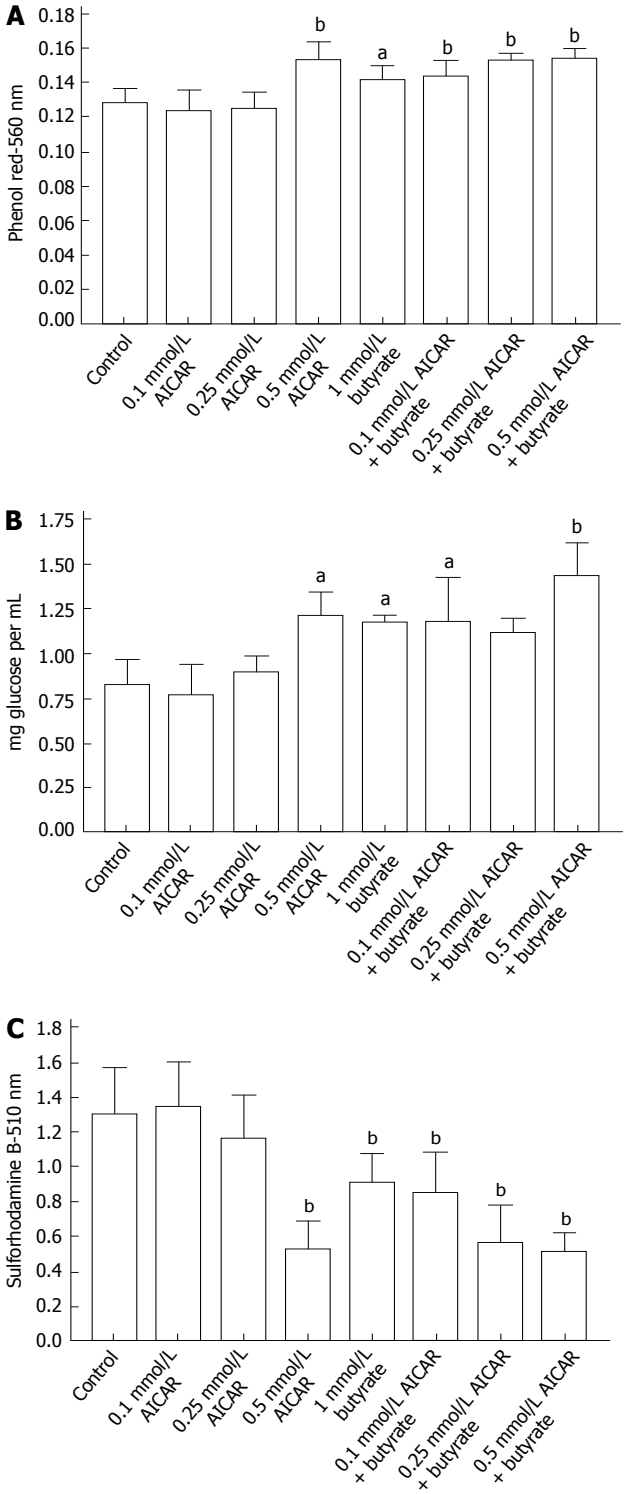


Figure 6 Effects of incubation of HT29 cells. Effects of incubation of HT29 cells for 72 h with butyrate and AICAR on acidification of the incubation medium (A), medium glucose concentration (B) and cell proliferation (C). $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control group.

4B). With the more rapidly dividing HCT116 wild type or p53 null cells there were significant decreases in medium acidification when cells were incubated with 0.5 mmol/L AICAR (Figure 5A) together with decreased glucose uptake (Figure 5B) and decreased cell proliferation (Figure 5C). Similarly with HT29 cells, AICAR at 0.1, 0.25 and 0.5 mmol/L caused the same trends (Figure 6).

In addition to effects of AMPK activators, inhibitory effects on medium acidification, glucose uptake, lactate production, reduction of Presto Blue and cell proliferation were also seen when the AMPK inhibitor, compound C, was incubated for 72 h with HCT116 cells (Figure 7).

Studies with several molecules that have been re-

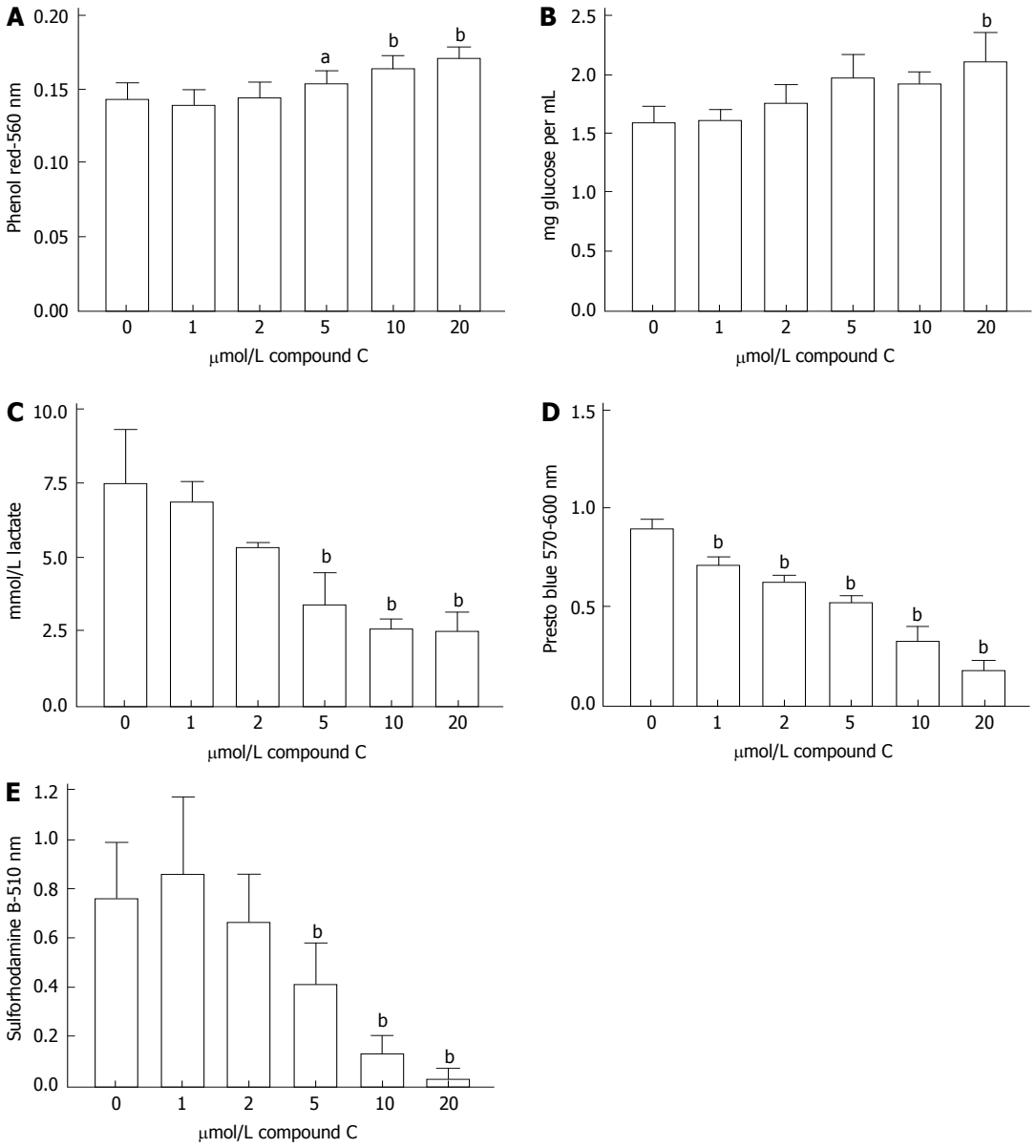


Figure 7 Effects of incubation of HCT116 WT cells. Effects of incubation of HCT116 WT cells for 72 h with compound C on acidification of the incubation medium (A), glucose concentration of the incubation medium (B), medium lactate concentration (C), reduction of Presto blue (D) and cell proliferation (E). $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control group.

ported to be associated with AMPK activation (salicylates, EGCG, KU-55933, quercetin and resveratrol) failed to reveal increased medium acidification and increased glucose uptake in colon cancer cells as previously established with metformin and phenformin[1]. The only exception was 5-aminosalicylic acid with which there were apparently lower glucose levels in the medium after incubation for 72 h (Figure 8A). This was surprising because the effect was not associated with increased medium acidification as seen with the biguanides and was seen at a concentration that did not result in significant inhibition of cell proliferation (Figure 8B). Further examination in the absence of cells revealed that the effect was an artifact due to inhibition of the enzyme-linked glucose assay. There was specificity for the effect because it was seen with 5-aminosalicylic acid but not with 4-aminosalicylic acid (Figure 9). The effect was seen with two samples of 5-aminosalicylic acid from Sigma-Aldrich, one containing 95% and the other containing 98% of the compound.

DISCUSSION

Our previous studies on the effects of metformin and phenformin on colon cancer cells revealed an unusual combination of effects[1]. These were an inhibition of cell proliferation despite an increase in glucose uptake and an increase in lactate production as monitored by acidification of the medium. Information in the literature suggests that biguanides may inhibit complex 1 of the mitochondrial transport chain and may result in activation of AMPK. The latter effect may not be direct and may be a consequence of increased levels of AMP and ADP or may be mediated through an upstream kinase, LKB1. We chose to examine the significance of AMPK activation on

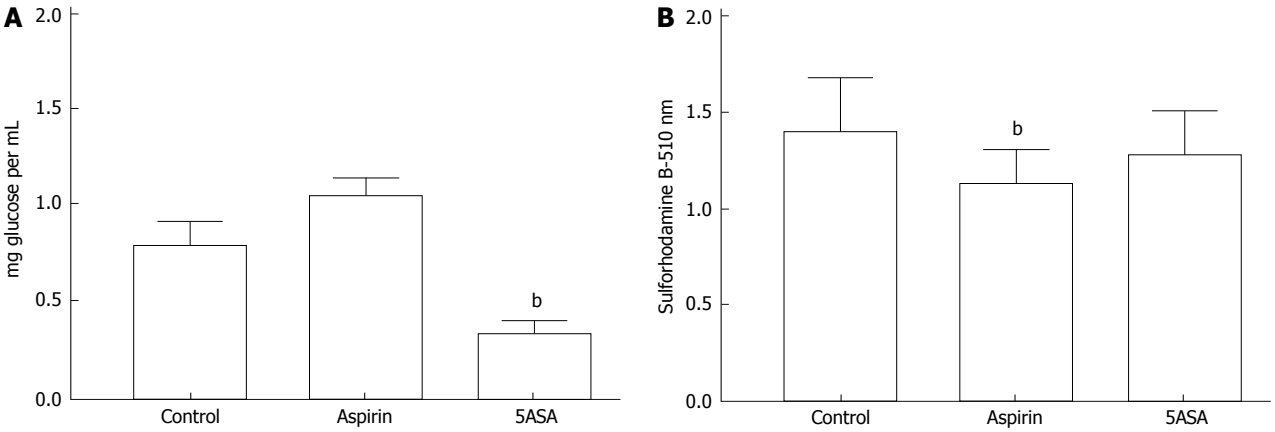


Figure 8 Effect of incubation of HT29 cells. Effect of incubation of HT29 cells for 72 h with 1 mmol/L acetylsalicylic acid (aspirin) and 1 mmol/L 5-aminosalicylic acid (5ASA) on glucose concentration of the incubation medium (A) and cell proliferation (B). $^{b}P < 0.01$ *vs* control group.

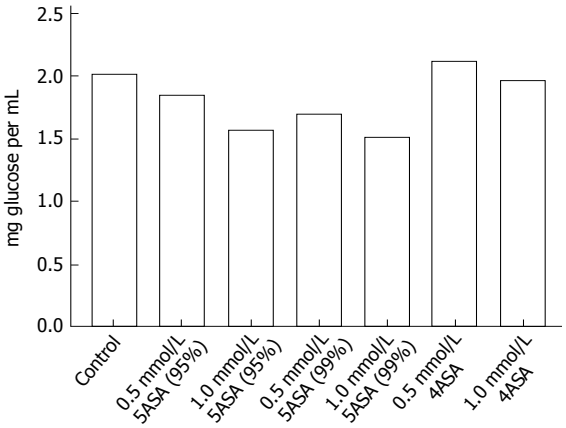


Figure 9 Effect of 5-aminosalicylic acid and 4-aminosalicylic acid. Effect of 5-aminosalicylic acid (5ASA) and 4-aminosalicylic acid (4ASA) on the assay of glucose in the RPMI 1640 medium that had not been incubated with colon cancer cells.

the metabolism and proliferation of colon cancer cells by studying the action of a variety of compounds reported to affect AMPK activity. The best characterized of these are A-769662[15] and AICAR[16]. These two compounds were found to be potential inhibitors of colon cancer cell proliferation but we observed neither an increase in glucose uptake nor increased medium acidification. To the contrary, decreased glucose uptake and decreased medium acidification was seen particularly with the more rapidly proliferating HT29 and HCT116 colon cancer cells.

Some of the compounds that were studied have been reported to be activators or inhibitors in different systems. Thus, quercetin has been reported to be an activator of AMPK[11] but Kim *et al*[17] found inhibition of AMPK by quercetin in HCT116 cells. Activation of AMPK by resveratrol has been reported[12,18] but Skrobuk *et al*[19] reported a situation in skeletal muscle where AMPK was inhibited by resveratrol. Compound C has consistently been found to be an inhibitor of AMPK. Although Compound C is a cell-permeable pyrrazolopyrimidine compound that can act as a reversible and ATP-competitive inhibitor of AMPK, actions on other target molecules have been reported[20]. We have extended our earlier studies with compound C and found that at concentrations frequently used to inhibit AMPK (1-10 μmol/L) it can be a potent inhibitor of colon cancer cell proliferation, most notably with HCT116 cells. Under those circumstances there was decreased glucose uptake and decreased acidification of the medium.

Potential chemopreventive action against colon cancer has been noted for some salicylates including acetylsalicylate[21] and 5-aminosalicylate[22]. At a concentration of 1 mmol/L we found that acetylsalicylic acid was a more potent inhibitor of colon cancer cell proliferation than 5-aminosalicylic acid. However, only with 5-aminosalicylic acid was there an apparent increase in glucose uptake. Further studies in the absence of cells indicated that this effect was due to interference with the enzyme-linked assay procedure for glucose. The assay uses a combination of glucose oxidase and peroxidase. It remains to be established whether one of these enzymes was more sensitive to the action of 5-aminosalicylic acid.

The tendency of cancer cells to show increased rates of glucose uptake and glycolysis even under aerobic conditions has become known as the Warburg effect. There is a paradox in that biguanides are of interest for their preventive or therapeutic action against cancer despite the observation that they seem to enhance the Warburg effect. The degree to which activation of AMPK relates to anti-cancer actions of biguanides remains an area of uncertainty[23-25]. It may be concluded from the present study that treatment with several agents that can affect AMPK activity results in the inhibition of the proliferation of colon cancer cells under conditions in which glucose metabolism is not enhanced.

COMMENTS

Background

Although there is a long history of the use of biguanides such as metformin in the treatment of type 2 diabetes, there is recent interest in the potential value of biguanides in the prevention and therapy of cancer. Rationale use of biguanides will be aided by comparison of their action with other compounds that can also

affect AMP-dependent protein kinase (AMPK) activity.

Research frontiers

Ongoing studies are investigating whether actions of biguanides on cancer cells relate to modulation of AMPK activity, effects mediated through inhibition of mitochondrial electron transport or changes in circulating insulin levels or combinations of these actions.

Innovations and breakthroughs

The observations described here emphasize that while biguanides and other compounds that modulate AMPK activity can affect the proliferation of cancer cells, there appears to be a unique pattern in the effects of metformin and phenformin that is also associated with increased glucose uptake and acidification of the extracellular environment.

Applications

The present work adds to the authors' knowledge of combined action of biguanides with other agents that may guide future combination therapies for the treatment of colon cancer. The results emphasize the need to better characterize actions of biguanides that relate to mechanisms other than modulation of AMPK activity.

Terminology

AMPK regulates metabolism so as to increase ATP production and limit ATP utilization. One potential mechanism of action for biguanides is to cause the upregulation of AMPK.

Peer review

The authors have investigated the inhibition of growth of colon cancer cells by compounds that affect AMPK activity but have divergent effect on metabolism. They have managed to show that treatment with several agents that can affect AMPK activity results in the inhibition of the proliferation of colon cancer cells under conditions in which glucose metabolism is not enhanced.

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RETROSPECTIVE STUDY

Prevalence and clinicopathological characteristics of appendiceal carcinoids in Sharjah (United Arab Emirates)

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Abstract

AIM: To determine the incidence and clinico-pathological profile of appendiceal carcinoids in a cohort of patients undergoing emergency appendicectomies for clinically suspected acute appendicitis in Sharjah, United Arab Emirates (UAE).

METHODS: The study included the retrospective data of 964 patients operated for clinically suspected acute appendicitis, and the resected specimens were received at Al-Qasmi Hospital (Sharjah) from January 2010 to December 2010. The data of the patients who were histologically reported to have carcinoid tumors of the appendix were extensively evaluated for the patient's demographics, indication for surgery, surgical procedure, tumor localization in the appendix, diameter of the lesion, concomitant appendicitis, immunohistochemistry studies and clinical follow-up.

RESULTS: Out of the 964 patients included in the study, 9 (0.93%) were found to have appendiceal carcinoids. The mean age reported was 28.7 years with a male to female ratio of 2:1. Eight tumors were located near the tip of the appendix with a mean diameter of 3.3 mm, while the remaining one was near the proximal end of the appendix. All the cases were associated with concomitant suppurative appendicitis. In seven reported cases, tumors were confined to the muscular layer while in one case each there was an extension to the serosa and mesoappendix, respectively. All tumors were found to be positive for chromogranin A, synaptophysin and neuron-specific enolase on immunohistochemistry but negative for cytokeratin-7. None of the patients developed recurrence or any reportable complications in the short follow-up period (12-26 mo) that was arranged as a six-monthly re-evaluation by abdominal ultrasonography.

CONCLUSION: Our study found a higher incidence of appendiceal carcinoids in patients undergoing emergency appendectomy for acute appendicitis in Sharjah, UAE compared to two previous studies from the Persian Gulf region. Interestingly, tumors were found to be more commonly in young males, which is in contrast to previous studies. Moreover, all the tumors were positive for common neuroendocrine markers.

Key words: Appendix; Carcinoid; Prevalence; Sharjah United Arab Emirates

Core tip: Incidence of appendiceal carcinoids is higher in patients undergoing emergency appendectomy for acute appendicitis in Emirate of Sharjah compared to two previous studies from the same geographical region. Moreover, tumors were found more commonly in

young males in contrary to previous studies.

Anwar K, Desai M, Al-Bloushi N, Alam F, Cyprian FS. Prevalence and clinicopathological characteristics of appendiceal carcinoids in Sharjah (United Arab Emirates). *World J Gastrointest Oncol* 2014; 6(7): 253-256 Available from: URL: http://www.wjgnet.com/1948-5204/full/v6/i7/253.htm DOI: http://dx.doi.org/10.4251/wjgo.v6.i7.253

INTRODUCTION

Carcinoid tumors are rare, slow-growing neuroendocrine tumors arising from the enterochromaffin cells disseminated throughout the gastrointestinal and bronchopulmonary systems[1]. The biological behavior of these tumors is poorly understood. Carcinoid tumors are considered indolent tumors as compared to adenocarcinoma, yet they have a potential to exhibit highly aggressive behavior. Although in 2004 they accounted for 1.25% of all malignancies, their frequency is augmenting by 6% annually[2]. In an American study the most common primary tumor site varied by race, with the lung being the most common in white patients, and the rectum as the most common site in Asian/Pacific Islander, American Indian/Alaskan Native, and African American patients[3].

The incidence of gastrointestinal carcinoids in both males and females has concurrently increased. A recent study from England analyzing the anatomic distribution of the tumors in 10324 cases revealed the commonest site to be the appendix, small intestine, colon, stomach and rectum in the decreasing order of frequency[4]. Additionally, the largest absolute increase in incidence of the carcinoid was also reported at the site of the appendix[4]. Recent data report the overall incidence of carcinoid tumors among patients undergoing emergency appendectomies between 0.27% and 1.6%[5,6].

Appendiceal carcinoid tumors are clinically silent and are usually an incidental finding in patients undergoing surgery for suspected acute appendicitis or during incidental appendectomy in the course of relevant abdominal surgery procedures[7]. Most appendiceal carcinoids are located at the tip of the organ. They are usually diminutive, measuring less than 1 cm, and rarely grow beyond than 2 cm in diameter[8]. Immunohistochemically carcinoid tumors of the gastrointestinal tract including the appendix express general neuroendocrine markers, such as chromogranin A, synaptophysin, non-specific enolase (NSE), CD56 and glucagon[9]. The gold standard treatment is surgical treatment by resection of the whole appendix for carcinoids located around the tip. In cases where the tumor is larger than 2 cm or located at the base of the appendix, a wider resection has to be performed with right hemicolectomy[1,2,4] .

The aim of the current study was to determine the incidence and clinicopathological characteristics of appendiceal carcinoids along with their immunohistochemical profile in a cohort of patients undergoing emergency appendectomies for clinically suspected acute appendicitis in Sharjah, United Arab Emirates (UAE).

Table 1 Clinicopathological characteristics of patients with appendiceal carcinoids from Emirates of Sharjah

Patient number[1]	Age (yr)	Gender	Tumor size (mm)	Extension[2]	Tumor localization
1	25	M	8	Serosal layer	28 mm from proximal end
2	29	M	4	Mesoappendix	Tip
3	33	M	4	Muscular layer	Tip
4	19	M	2	Muscular layer	2 mm from tip
5	28	M	1	Muscular layer	Tip
6	54	M	1	Muscular layer	6 mm from tip
7	25	F	4	Muscular layer	13 mm from tip
8	18	F	3	Muscular layer	Tip
9	27	F	3	Muscular layer	10 mm from tip

[1]All cases underwent open appendectomy for clinical diagnosis of appendicitis which was further confirmed on microscopic examination; [2]No vascular invasion was identified in any case. M: Male; F: Female.

MATERIALS AND METHODS

This retrospective study was carried out at the Pathology Department of Al-Qasmi Hospital, Sharjah, UAE, which is the only tertiary care government facility in the region for the histopathological analysis of the surgical specimens. This study includes all consecutive patients who underwent appendectomies between January 2010 and December 2010 in Sharjah, UAE, and their specimens were received at the hospital for analysis. Only the data of the patients who were histologically reported to have carcinoid tumors of the appendix was reviewed for the patient's age, gender, indication for surgery and surgical procedure. The histological analysis included tumor localization in the appendix, evaluation of the diameter of the lesion after fixation with formaldehyde, concomitant appendicitis, and immunohistochemical analysis of chromograninin A, synaptophysin, NSE, serotonin, carcinoembryonic antigen (CEA), CK-7 and cytokeratin-20 (CK-20). Patient follow-up was conducted for those diagnosed with carcinoids only every 6 mo and recurrence evaluated by abdominal ultrasonography.

RESULTS

Nine hundred and sixty-four patients underwent appendectomies during the study period, of whom 9 (0.93%) were found to have histological evidence of carcinoid tumors of the appendix. The clinicopathological data in relation to carcinoids are shown in Table 1. There were 6 male and 3 female patients with a mean age of 28.7 years (range, 18-54 years). All the cases were operated for a clinical suspicion of appendicitis. Histologically 4 carcinoid lesions were demonstrated at the tip, another 4 ranged from 2-13 mm away from the tip and one lesion was located 28 mm from the base of the appendix. The

Table 2 Immunohistochemical characterization of appendiceal carcinoid tumors in patients from Emirates of Sharjah

Patient number	Age (yr)	Sex	CG	Synaptophysin	NSE	5-HT	CEA	CK20	CK7
1	25	M	+	+	+	-	-	-	-
2	29	M	+	+	+	+	-	-	-
3	33	M	+	+	+	-	-	-	-
4	19	M	+	+	+	-	-	+	-
5	28	M	N/D	N/D	N/D	N/D	N/D	N/D	N/D
6	54	M	+	+	+	+	+	-	-
7	25	F	+	+	+	+	-	-	-
8	18	F	+	+	+	-	-	-	-
9	27	F	+	+	+	+	-	-	-

CG: Chromogranin; NSE: Non-specific enolase; 5-HT: Serotonin; CEA: Carcinoembryonic antigen; CK-20: Cytokeratin 20; CK-7: Cytokeratin 7; N/D: Not determined as the tissue sample was unavailable for the staining procedure; M: Male; F: Female.

mean diameter of the tumors was 3.3 mm (range, 1-8 mm). Concomitant suppurative appendicitis was present in all cases. Seven tumors were confined to the muscular layer, while one case exhibited an extension to the serosa and another extended to the mesoappendix. The margins of all the resected tissue samples received for histological analysis, however, were free of tumor cells.

In one case the tissue sample from the tip was very infinitesimal to be evaluated by immunohistochemistry (IHC). The rest eight tumors were positive for chromogranin A, synaptophysin and NSE as shown in Table 2. Four tumors were additionally found to be positive for serotonin and one each for CEA and CK-20. None of the tumors was positive for CK-7.

All patients remained disease-free after a median follow-up duration of 22 mo (range, 12-26 mo).

DISCUSSION

Carcinoid tumors were not considered to be common tumors, but recent studies suggest an abrupt increase in their incidence and prevalence over the last few decades. Additionally, the appendix has been identified as one of the most common sites for carcinoids in the gastrointestinal tract[3,10]. The reason for this rise remains, as yet, obscure, although an increase in the number of elective appendectomies was considered to be one of the contributing factors. Contrary to this belief, a recent study demonstrated that the number of surgeries did not actually influence the incidence of appendiceal carcinoids[6]. However, more extensive pathological examination including multiple sections from different parts of the appendix may have played a part in detecting even the tiny foci of the tumors. Our present findings validate this hypothesis since most of the carcinoids identified were relatively small in size (1-4 mm in diameter). Carcinoid tumors are generally asymptomatic due to their small size and specific location in the appendix and are commonly diagnosed as an incidental finding in emergency or elective appendectomy specimens[11]. Although the majority of the carcinoids exhibit benign behavior, they do have a malignant potential with the ability to metastasize[7].

Our present study reports the incidence of carcinoid tumors at 0.93% per annum in the pathological specimens obtained during emergency appendectomies. This incidence is quite high compared to that reported by two other studies conducted in the same geographical region. The reported incidence in appendectomy specimens from Iran was 0.2% and that from Saudi Arabia 0.6%[12,13]. However, in most studies from other geographical regions the incidental histological diagnosis of carcinoid ranged from 0.3%-0.9% in patients undergoing appendectomy[8]. In a recent study conducted in a community teaching hospital in South Australia, appendiceal carcinoids were even found to occur in 1.6% of emergency appendectomies performed for acute appendicitis[6].

We did not observe a female preponderance in our patients with carcinoids as suggested in many previous studies[12-14]. We are unable to explain this gender disparity in our study where males were affected by this neoplastic lesion twice as frequently as females. There may be, however, a strong environmental bias in the UAE for this discrepancy. The gut microbiome influences both the development of the mucosal immune system as well as the regulation of epithelial regeneration[15]. Previous literature has indicated carcinoid tumors to be distributed among younger age groups (20-30 years of age) and their preferential location in the tip of the appendix, with the latter being attributed to the increased density of subepithelial neuroendocrine cells near the tip[16,17]. Our observations in the present study confirm these findings (Table 1). The average age for males was 31.3 years while for females it was 23.3 years. The mean overall age of the patients was 28.7 years.

Approximately 80% of appendiceal carcinoids are less than one centimeter in diameter[8]. Our present findings are consistent with previous studies as the tumor size in all cases in our study were less than one centimeter, with eight cases measuring between 1 and 5 mm and one 8 mm in diameter. Seven carcinoids were confined to the muscular layer, while one extended into the serosal layer and another one was located in the mesoappendix (Table 1).

All carcinoid tumors evaluated in this series showed positive IHC staining for common neuroendocrine markers. Interestingly, all the samples identified were positive for chromogranin A, synaptophysin and neu-

ron-specific enolase (Table 2). However, four carcinoids were positive for serotonin and one each for CEA and CK-20, respectively, all of them had a size between 1-4 mm. A previous study has demonstrated variable staining for these markers (62%-85%) in gastrointestinal carcinoinds[9]. The staining characteristics observed in our study were not associated with any other clinicopathological characteristics.

Although some carcinoids have been reported to be aggressive, none of the patients had recurrence or any reportable complications in the short follow-up period (12-26 mo). Histological analysis of the draining lymph nodes or the liver was not performed due to gross normal appearance and unremarkable abdominal ultrasonographic findings in these patients. The metastatic potential of carcinoids cannot be accurately assessed based on the follow-up duration, and this is a limitation of the current study.

Our seminal study from this region shows the incidence of appendiceal carcinoids in patients undergoing emergency appendectomies for clinically suspected acute appendicitis from Sharjah, UAE to be higher than that reported by two previous studies from the same geographical region. Contrary to other studies, young males were involved two times more commonly than the females. All tumors were found positive for common neuroendocrine markers.

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COMMENTS

Background

Carcinoid tumors are considered to be one of the commonest tumors in the appendix. Their incidence has been shown to vary in different studies and this seminal study details the prevalence of these tumors in the United Arab Emirates.

Innovations and breakthroughs

This is the first study from the region that shows that the incidence of appendiceal carcinoid tumors has augmented as compared to the previous studies from the region. Interestingly, this rise is observed in the young male population instead of the females, as highlighted in previous studies.

Applications

Such a difference in incidence necessitates an investigative research into the etiology and further monitoring to evaluate the trend of these tumors that may be associated with environmental factors due to changes in the gut microbiome. Repetitive evaluations are fundamental to assess incidence rates in cancer demographics. In addition data from other countries in the Persian Gulf region can provide a better global perspective.

Peer review

The authors present a subject of importance for the surgical community: the carcinoids of the appendix.

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PROSPECTIVE STUDY

Patient prompting of their physician resulted in increased colon cancer screening referrals

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Abstract

AIM: To determine whether a communication instrument provided to patients prior to their primary care physician (PCP) visit initiates a conversation with their PCP about colorectal cancer screening (CRC-S), impacting screening referral rates in fully insured and underinsured patients.

METHODS: A prospective randomized control study was performed at a single academic center outpatient internal medicine (IRMC, underinsured) and family medicine (FMRC, insured) resident clinics prior to scheduled visits. In the intervention group, a pamphlet about the benefit of CRC-S and a reminder card were given to patients before the scheduled visit for prompting of CRC-S referral by their PCP. The main outcome measured was frequency of CRC-S referral in each clinic after intervention.

RESULTS: In the IRMC, 148 patients participated, a control group of 72 patients (40F and 32M) and 76 patients (48F and 28M) in the intervention group. Referrals for CRC-S occurred in 45/72 (63%) of control *vs* 70/76 (92%) in the intervention group ($P \leqslant 0.001$). In the FMRC, 126 patients participated, 66 (39F:27M) control and 60 (33F:27M) in the intervention group. CRC-S referrals occurred in 47/66 (71%) of controls *vs* 56/60 (98%) in the intervention group ($P \leqslant 0.001$).

CONCLUSION: Patient initiated physician prompting produced a significant referral increase for CRC-S in underinsured and insured patient populations. Additional investigation aimed at increasing CRC-S acceptance is warranted.

Key words: Colon cancer; Screening; Primary care; Physician patient relationship; Referral

Core tip: Colon cancer screening only performed in approximately 60% of Americans over 50 years old. Inadequate communication between patient and physician is a significant obstacle to obtaining appropriate screening, especially in the underinsured population. Patient initiated prompting of their primary care physician for colorectal cancer screening with colonoscopy increased referrals in both underinsured and insured patient groups.

Le V, Syed S, Vega KJ, Sharma T, Madhoun MF, Srinivasan N, Houchen CW. Patient prompting of their physician resulted in increased colon cancer screening referrals. *World J Gastrointest Oncol* 2014; 6(7): 257-262 Available from: URL: http://www.wjgnet.com/1948-5204/full/v6/i7/257.htm DOI: http://dx.doi.org/10.4251/wjgo.v6.i7.257

INTRODUCTION

In spite of the available evidence suggesting effectiveness of colorectal cancer screening (CRC-S), approximately 50% of the United States population over 50 years old has not had CRC-S[1]. According to the National Cancer Institute, in 2009 the estimated new cases of colon cancer and rectal cancer in United States were 106100 and 40870 respectively. The estimated death of these combined cancers was 49920 (www.cancer.gov). Several studies have been conducted to understand the barriers for colorectal screening[2]. Inadequate communication between the primary care physician (PCP) and patient, including lack of a physician's recommendation for testing and patients unawareness were found to be important barriers[2-4]. Other investigators have shown colonoscopy as a safe and feasible primary screening test[5]. In addition, studies have also shown that in average risk patients, colonoscopy screening found 0.5%-1.0% have colon cancers and 5%-10% have advanced neoplasia that can be removed during the screening[5-9]. Providing educational material and a method for the patient to express interest in CRC-S to their PCP could increase referral for this screening. The aim of our study was to determine if patient initiated prompting of their PCP for CRC-S would increase referrals in both underinsured and insured patients.

MATERIALS AND METHODS

From November 2008 to November 2010, all patients seen in Family Medicine Resident Clinic (FMRC, insured) and Internal Medicine Resident Clinic (IMRC, underinsured) waiting areas were screened for CRC-S eligibility. Those patients meeting criteria for screening but never having been screened previously were considered eligible for the study. Eligible patients were assigned randomly to either a control or intervention group. Intervention consisted of a pamphlet describing the benefit of CRC-S, given to patients prior to their PCP visit and a reminder note about CRC screening to be given to their physician during the encounter. The pamphlet discussed colon cancer incidence, frequency, deaths, prevention, need for screening, risk factors, symptoms, available screening methods with colonoscopy preferred based on ACG guidelines. In order to not reveal the purpose of our study to resident physicians, patients were randomly assigned as control group or intervention group on different clinic days. Since, each resident physician only see patients on one specific day of clinic, and by randomizing patients on the same day will allow the physicians to figure out our study if he received a reminder note on one patient and not the other. A two-page questionnaire was designed to assess the referral patterns and preferred screening method for CRC. Questions on the survey included demographic parameters (age, race, gender, and education level), whether their PCP had referred them for CRC-S, the screening method recommended, whether the participants accepted the screening referral, presence of insurance coverage for CRC-S, and knowledge that CRC could be prevented using screening. Upon completion of the study, all patients in the control group were given the CRC-S pamphlet for use.

The primary outcome was to determine if patient-initiated prompting for CRC-S of their primary care physicians increased CRC-S referrals. We wanted to determine if a communication instrument provided to patients initiated a conversation with their primary care physicians about CRC screening, especially *via* colonoscopy. The secondary outcome was to determine whether differences exist in regard to patient-physician communication patterns about screening among residents and faculty in the general internal medicine and family practice clinics. We were also interested in the method of CRC-S given to the patients and the overall acceptance rates for CRC-S among patients.

Statistical analysis

The minimum sample size required to detect a referral frequency difference of 25% after patient initiated prompting was calculated using a confidence level of 95% and confidence interval of 5%. The sample size needed for each group was 52 patients. Differences between groups were analyzed using the unpaired Student's *t*-test for normally distributed data or the Mann-Whitney *U* test for skewed data. The χ^2 test was used for comparisons of categorical variables. Multivariate analysis using stepwise logistic regression was performed to identify independent factors associated with CRC-S referral. All statistical analysis was done using SAS software (v 9.1.3, SAS Institute, Cary, NC). All statistical tests were carried out at an alpha of 0.05.

RESULTS

A total of 274 patients were included from both clinic sites in the present investigation. One hundred forty eight (148) patients were seen in the IMRC and 126 were seen in the FRMC (Figure 1). Among the IRMC patients, 72 (40F:32M) were in the control group and 76 (48F:28M) in the intervention group. In the FRMC patients, 66 (39F:27M) were in the control group and 60 (33F:27M) in the intervention group. No differences were observed in baseline parameters of control or intervention groups from either of the 2 clinics (Table 1). Patient initiated prompting of PCP (intervention) resulted in a significant referral increase for CRC-S in both underinsured and insured patient populations. In the IMRC, 63% in the control group (45/72) got referrals for CRC-S *vs* 92% in

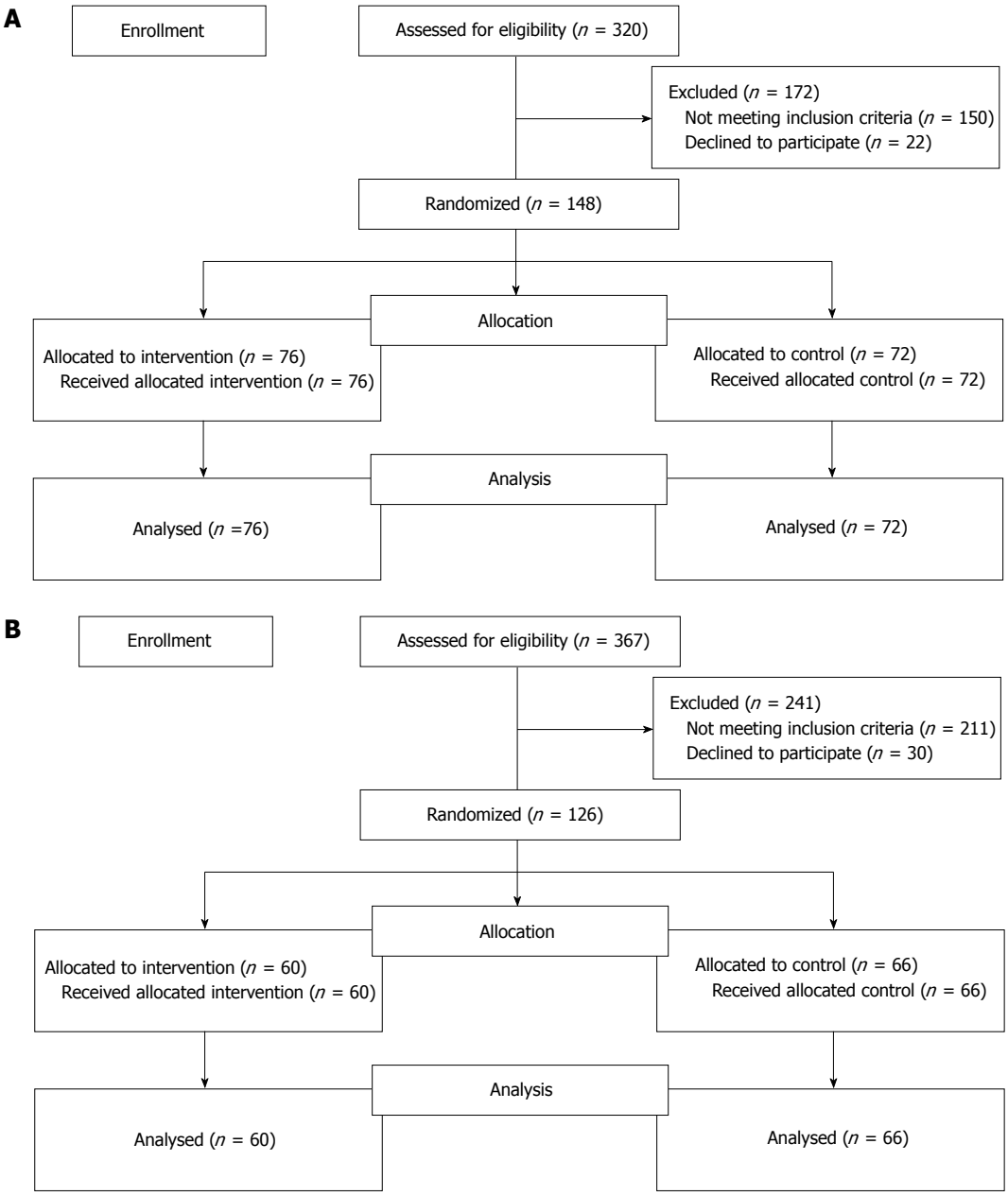

Figure 1 Patient distribution in both clinics between intervention and control groups. A: Internal medicine resident clinic (underinsured); B: Family medicine resident clinic (insured).

the intervention group (70/76, $P \leqslant 0.001$, Figure 2A). In the FMRC, 47/66 (71%) in the control group were referred for CRC-S *vs* 56/60 (98%) in the intervention group ($P \leqslant 0.001$, Figure 2B).

No difference was seen in referral acceptance between the 2 clinics. Among those who got referrals for CRC-S in the IMRC, 31/45 (69%) in the control group *vs* 41/70 (59%) in the intervention group accepted the referrals, (P = NS, Figure 2A). In patients from FMRC who were referred for CRC-S, 36/47 (77%) in the control group *vs* 41/56 (73%) in the intervention group accepted the referral, (P = NS, Figure 2B). In univariate analysis, factors related CRC-S referrals were having insurance (60% *vs* 46%, $P = 0.045$), male gender (38% *vs* 54%, $P = 0.027$), knowledge of CRC recommendations (46% *vs* 26%, $P = 0.0085$) and patients initiated promoting of PCP (intervention) (58% *vs* 18%, $P < 0.0001$). On multivariate logistic regression analysis, male gender (OR = 0.49, 95%CI: 0.26-0.93, $P = 0.03$) and patient initiated promoting the PCP (OR = 6.3, 95%CI: 2.9-13.2, $P < 0.0001$) were identified as independent predictors (Table 2).

All patients referred for CRC-S were offered colonoscopy as the only screening method. Patients were not advised of any other CRC-S method after declining colonoscopy. Overall, 37% of participants in the IMRC and 35% in the FRMC declined CRC-S recommended by the physicians. The primary issue influencing patients' decision to defer CRC-S referral was financial difficulty. Bowel preparation fear, procedure related complications, unsure of colonoscopy benefit, and concern of finding cancer were other, less frequent reasons for not accepting CRC-S referral (Figure 3).

Table 1 Patients characteristics

Characteristics	IMRC		*P* value	FMRC		*P* value
	Control group	Intervention group		Control group	Intervention group	
Number of patients	72	76		66	60	
Median age (range), yr	54 (51-64)	56 (49-70)		55 (48-68)	54 (47-66)	
Sex						
Male	32	28		27	27	
Female	40	48		39	33	
Ethnicity						
Non-hispanic white	35	45		30	32	
African American	26	24		25	19	
Others	9	9		11	9	
Health Insurance						
Yes	12	19		66	60	
No	60	57		0	0	
Education						
< High school graduate	12	25		7	5	
High school graduate	54	41		29	31	
College graduate	6	10		30	24	
Past medical history						
Hypertension	56	60		51	37	
Diabetes mellitus	31	26	NS	25	21	NS
Heart disease	4	7		5	5	
Liver disease	6	6		5	3	
None	12	6		3	9	
Alarm symptoms						
Yes	20	33		28	26	
No	52	43		38	34	
Family history of CRC						
Yes	11	4		12	7	
No	61	72		54	52	
Had a colonoscopy						
Yes	6	11		9	8	
No	66	65		57	52	
Knowledge of CRC recommendations						
Yes	14	26		36	38	
No	58	50		30	22	
Know colonoscopy prevents CRC						
Yes	35	46		42	42	
No	37	30		24	18	

CRC: Colorectal cancer; IMRC: Internal medicine resident clinic; FMRC: Family medicine resident clinic.

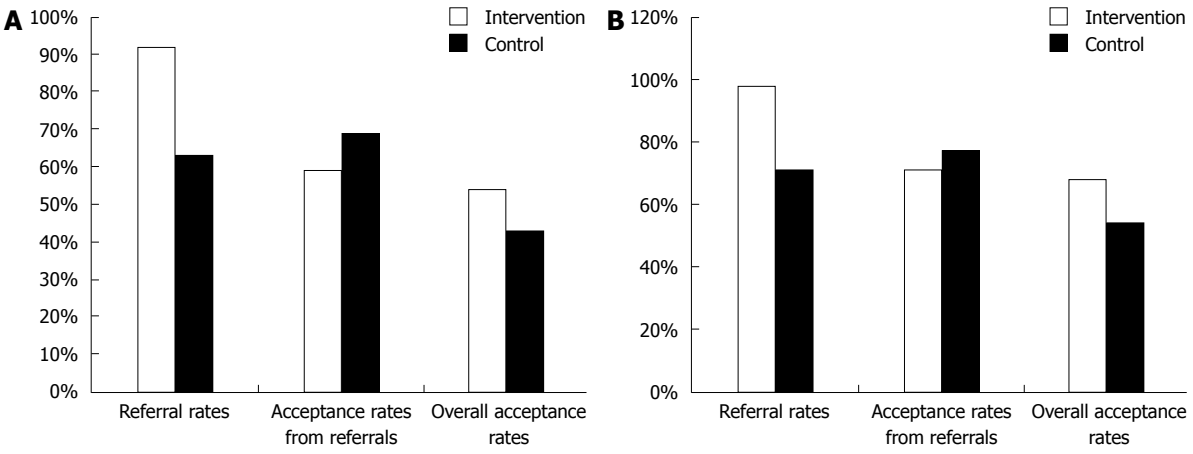

Figure 2 Patterns of referral and acceptance. A: In internal medicine resident clinic (underinsured patients); B: Family medicine resident clinic (Insured patients).

DISCUSSION

Colorectal cancer is the fourth most common cancer diagnosed and second leading cause of cancer related death in the United States[1]. Early stage detection of colorectal cancer has a survival rate of around 80%[1]. Despite the proven efficacy of colorectal cancer screening, only about 50% of eligible patients in the United States are currently

Table 2 Univariate and multivariate analysis of factors impacting colon cancer screening referral *n* (%)

	Offered CRC screening (*n* = 210)	Not offered CRC screening (*n* = 54)	*P* value
Age, mean ± SD	55 ± 4	55 ± 4	0.810
White race	116 (55)	26 (48)	0.350
Male sex	83 (38)	31 (54)	0.027
Higher education	57 (26)	13 (23)	0.590
Insured	131 (60)	26 (46)	0.045
Limiting medical problems	23 (11)	5 (8)	0.680
Symptomatic	91 (42)	16 (28)	0.056
Family history	23 (11)	11 (19)	0.076
Knowledge of CRC recommendations	99 (46)	15 (26)	0.0085
Received pamphlet	126 (58)	10 (18)	< 0.0001
Family medicine providers	102 (47)	24 (42)	0.510

CRC: Colorectal cancer.

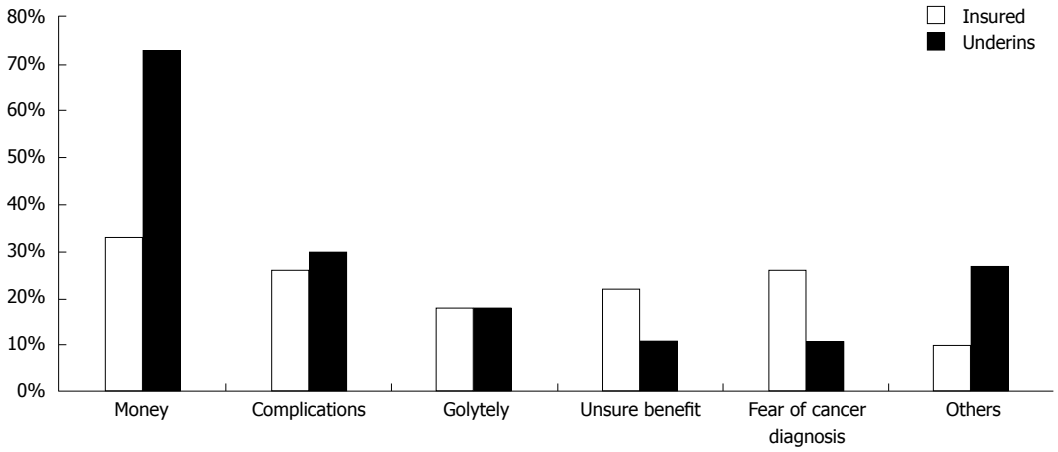

Figure 3 Factors resulting in declining referral between insured and underinsured patients. Underins: Underinsured patients.

being screened[1]. Effective interventions as attempts to increase the referral for CRC-S are lacking. Studies have identified that a lack of communication between physicians and patients was the most common factor resulting in inadequate referrals for CRC-S[2-4]. However, few studies focus on the patient as a factor that contributes to this issue. The primary outcome of our study was to determine if patient initiated prompting of their PCP for CRC-S would increase referrals in both underinsured and insured patients. Increasing patient awareness combined with PCP prompting by patients about CRC-S resulted in increased referral rates.

Among the intervention groups in both clinics, ethnicity did not appear to impact the frequency of patient prompting of physician for CRC-S (data not shown). It is well known that African Americans do not get CRC-S as frequently as non-Hispanic whites[10]. This intervention may help narrow the CRC-S disparity observed, improving long term outcome from this disease.

Multiple barriers to colorectal cancer screening referral by PCPs have been identified in the literature[11-15]. The present study reveals another method where PCPs can be reminded of patient interest in CRC-S and provide appropriate referral for the procedure. This type of intervention using patient prompting of their PCP could decrease the burden on the PCP to remember appropriate CRC-S recommendations, resulting in an increased screening rate overall.

Referral rates after intervention were found to be increased in both clinic populations but acceptance rates after referral were less in both intervention groups, unexpectedly. This resulted in lower overall acceptance rates for both clinics and was not significantly different between intervention or control groups. Multiple factors have been identified which contribute to a reduced acceptance rate for CRC-S[16]. In our study, multiple issues were evident. First, college education was more prevalent in patients with medical insurance coverage and more of these individuals were aware of current CRC-S literature than underinsured patients. However, this did not impact whether CRC screening was offered. Secondly, we observed a higher acceptance rate, in insured patients, for CRC-S offered by their primary physicians compared to the underinsured which has been reported by previous investigators[17-19]. Finally, acceptance rate for CRC-S was increased in patients with alarm symptoms compared to asymptomatic patients in both control and intervention groups. The most common limiting factor influenced patient's decision to refuse CRC screening was financial affordability in both underinsured (72%) and insured

populations (36%) even though significantly lower in the insured population. Procedure complications, bowel preparation concerns, colonoscopy benefit uncertainty, and fear of finding cancer were other less common reasons for not accepting referrals.

A limitation to the present study is not using other screening methods available if colonoscopy is declined. As colonoscopy was considered the test of choice and other methods, if positive, result in colonoscopy referral, use of alternative screening tools appeared redundant to the investigators. However, some individuals may prefer colonoscopy only following a positive result from another screening tool and should be considered in larger scale investigations.

CRC-S referrals significantly increased with patient initiated prompting of physicians for such screening. Larger investigations, using this method, directed towards increasing acceptance of CRC-S are warranted.

COMMENTS

Background

Despite the available evidence suggesting the effectiveness of colorectal screening (CRC-S), almost half of the United States population over 50 years has not been tested. According to the National Cancer Institute, in 2009 the estimated new cases of colon cancer and rectal cancer in United States were 106100 and 40870 respectively.

Research frontiers

Effective interventions to increase patient referrals for CRC-S are lacking. Studies have identified that a lack of communication between physicians and patients was the most common factor resulting in inadequate referrals for CRC-S.

Innovations and breakthroughs

As colonoscopy was considered the test of choice and other methods, if positive, result in colonoscopy referral, use of alternative screening tools appeared redundant to the investigators.

Peer review

This is a well constructed study, of high clinical significance. It seems that it is sufficiently powered to detect pre-specified 25% difference in referral frequency, but in my opinion this sample size is not sufficiently enough to portray independent predictors resulting in declining referral between insured and underinsured patients.

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TOPIC HIGHLIGHT

Dr. Edward Yu, *Series Editor*

Esophageal cancer management controversies: Radiation oncology point of view

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Abstract

Esophageal cancer treatment has evolved from single modality to trimodality therapy. There are some controversies of the role, target volumes and dose of radiotherapy (RT) in the literature over decades. The present review focuses primarily on RT as part of the treatment modalities, and highlight on the RT volume and its dose in the management of esophageal cancer. The randomized adjuvant chemoradiation (CRT) trial, intergroup trial (INT 0116) enrolled 559 patients with resected adenocarcinoma of the stomach or gastroesophageal junction. They were randomly assigned to surgery plus postoperative CRT or surgery alone. Analyses show robust treatment benefit of adjuvant CRT in most subsets for postoperative CRT. The Chemoradiotherapy for Oesophageal Cancer Followed by Surgery Study (CROSS) used a lower RT dose of 41.4 Gray in 23 fractions with newer chemotherapeutic agents carboplatin and paclitaxel to achieve an excellent result. Target volume of external beam radiation therapy and its coverage have been in debate for years among radiation oncologists. Pre-operative and post-operative target volumes are designed to optimize for disease control. Esophageal brachytherapy is effective in the palliation of dysphagia, but should not be given concomitantly with chemotherapy or external beam RT. The role of brachytherapy in multimodality management requires further investigation. On-going studies of multidisciplinary treatment in locally advanced cancer include: ZTOG1201 trial (a phase Ⅱ trial of neoadjuvant and adjuvant CRT) and QUINTETT (a phase Ⅲ trial of neoadjuvant *vs* adjuvant therapy with quality of life analysis). These trials hopefully will shed more light on the future management of esophageal cancer.

Key words: Radiotherapy; Chemotherapy; Esophagus; Cancer; Treatment

Core tip: Esophageal cancer treatment has evolved from single modality to trimodality therapy. There are some controversies of the role, target volumes and dose of radiotherapy (RT) in the literature over decades. Esophageal brachytherapy is effective in the palliation of dysphagia, but should not be given concomitantly with chemo or external beam RT. On-going studies include: ZTOG1201 trial (a phase Ⅱ trial of neoadjuvant and adjuvant chemoradiation) and QUINTETT (a phase Ⅲ trial of neoadjuvant *vs* adjuvant therapy). These trials hopefully will shed more light on the future management of esophageal cancer.

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INTRODUCTION

Over the past 20 years there have been many significant

changes in the management of esophageal cancer. This disease has shown remarkable changes in histology of adenocarcinoma on the rise over squamous cell carcinoma, and in epidemiology with concentration of tumors adjacent to the gastro-esophageal junction (GEJ). Esophageal cancer has evolved from single modality treatment in the past to trimodality treatment currently. Radiotherapy (RT) has been part of the integral management of esophageal cancer for decades. Greater understanding of the natural history has influenced the approach to diagnosis and to treatment options. Appreciation of the need for multidisciplinary approach in treatment planning has reflected the important role of various treatment modalities. There are different clinical practices of combined treatments and controversies often arise. This is aggravated by the difficulty to conduct large-scale randomized trials since many patients are elderly with multiple co-morbidities. A Medline search revealed a limited number of randomized studies in the past decade. The present article reviews RT in the multimodality management of esophageal cancer, with emphasis on the controversy of RT target volume, and radiation dose. A few examples of the controversies are listed here in this section.

The challenges to treat elderly patients with esophageal cancers had been reported[1]. During recent years, the curative potential of RT *vs* surgery for esophageal cancer was investigated in randomized trials. A metaanalysis showed that overall survival (OS) was equivalent between surgery and definitive chemoradiotherapy (CRT) (HR = 0.98 95%CI: 0.8-1.2, P = 0.84)[2]. There was a trend to more cancer related deaths in the definitive RT+/-chemotherapy (chemo) arms [HR = 1.19 (0.98-1.44), P = 0.07], predominantly due to a higher risk of loco-regional progression [HR = 1.54 (1.2-1.98), P = 0.0007] but treatment related mortality was lower in the conservative arms [HR = 0.16 (0-0.89), P = 0.001]. The similar outcome in survival suggests that the safer approach of CRT is a reasonable choice especially in comorbid patients with esophageal squamous cell carcinoma.

For patients with less advanced esophageal cancer patients, the benefit of neoadjuvant therapy is still unclear. However, due to the significant under staging of T2 N0 patients (50% in the Johns Hopkins series), the authors recommend neoadjuvant therapy to all cT2N0 patients before operation[3].

ROLE OF EXTERNAL BEAM RT

Surgery has been considered the standard of care for stage I resectable esophageal cancer with 5 year survival of 60%-70%, stage Ⅱ 40%, stage Ⅲ 20%[4]. RT will be discussed in the following sections including its role with chemo before surgery (abbreviated as S here), after surgery with and without chemo, and whether RT is needed in the trimodality management: (1) C + S *vs* S; (2) CRT + S *vs* S; (3) S *vs* S + RT; (4) S *vs* S + CRT; (5) CRT + S *vs* S + CRT; and (6) CRT + S *vs* CRT.

C + S vs S: Perioperative chemo without RT

A landmark study confirmed that this treatment improves survival. The 503-patient United Kingdom National Cancer Research Institute Medical Research Council Adjuvant Gastric Infusional Chemo trial is the first randomized trial to demonstrate a conclusive survival benefit of perioperative chemo for patients with resectable adenocarcinoma of the stomach, GEJ, and lower esophagus, compared with surgery alone[5]. Epirubicin, cisplatin, and infused 5-fluorouracil (ECF) decreased tumor size and stage and hence significantly improved progression-free and overall survival. However, infusional chemo is difficult to administer[6]. In this study, RT is not required. Opinions arise regarding the relative efficacy of CRT *vs* chemo alone in the multimodality management setting. A multicenters randomized Trial of Preoperative therapy for Gastric and Esophagogastric Junction Adenocarcinoma from National Cancer Institute of Canada, European Organization for Research and Treatment of Cancer (EORTC), and Trans-Tasman Radiation Oncology Group is underway to compare preoperative CRT using 45 Gray (Gy) with preoperative chemo alone for GEJ and gastric adenocarcinoma[7]. The chemo regimen in both arms is ECF or EC Xeloda. The result of this trial may offer further insight to the above dilemma that clinicians often have.

CRT + S vs S: Does neoadjuvant CRT improve survival?

The use of neoadjuvant CRT has become an increasingly used treatment approach[8]. Tables 1 and 2 summarizes the potential benefit of preoperative therapy[9]. A few key randomized clinical trials of preoperative CRT with surgery compared to surgery alone are discussed below. Caution to compare across studies is advised. There is great variation of RT dose schemes and the optimum treatment schedule is not clear.

Nygaard *et al*[10] showed that 3-year survival was significantly higher in the pooled groups receiving RT as compared with the pooled groups not receiving RT. Comparison of the groups having pre-operative chemotherapy with those not having chemo showed no significant difference in survival.

Walsh *et al*[11] employed two courses of 5-flurouracil (5-FU), 15 mg/kg daily for five days, and cisplatin, 75 mg/m^2 on day 7. This cycle was repeated in week 6. RT of 40 Gy/15 fractions (f)/3 wk was administered.

Bosset *et al*[12] with the Fondation Française de Cancérologie Digestive and EORTC Gastrointestinal Tract Cancer cooperative Group conducted the largest study of its kind with 282 patients. They gave two courses of cisplatin, at a dose of 80 mg/m^2 on 0 to 2 d before each course of RT. The target of RT was the macroscopic tumor and enlarged lymph nodes, if any, surrounded by 5-cm proximal and distal margins and a 2-cm radial margin. After a median follow-up of 55.2 mo, no significant difference in OS was observed; the median survival was 18.6 mo for both groups. Although median or OS

Table 1 Important randomized trials for preoperative chemoradiation *n* (%)

Ref.	*n*	Histology	Treatment	R0	pCR	Op mortality	MS	3 YS	Locoregional failure
Nygaard *et al*[10], 1992		Sq	S	37%	-	5 (3.4)	Approximately 0.6 yr	Approximately 9%	-
			CB → S	41%		6 (4.0)	Approximately 0.7 yr	Approximately 2%	
			R → S	40%		4 (2.7)	Approximately 0.9 yr	Approximately 20%	
			CB + R → S	55% (Gp 4 *vs* 1, *P* = 0.08)		8 (5.4)	Approximately 0.7 yr	Approximately 18%	
Walsh *et al*[11], 1996	113	A	CF + R → S	-	25%	5 (10.4)	16	32%	-
			S	-	0%	2 (3.7)	11 mo	6%	-
							P = 0.01	*P* = 0.01	
Bosset *et al*[12], 1997	282	Sq	C + R → S	-	26%	17 (12.3)	18.6 mo	36%	-
			S	-	0%	5 (3.6)	18.6 mo	34%	-
								See text	
Urba *et al*[13], 2001	100	75% A	CFV + R → S	90%	28%	1 (2.1)	16.9 mo	30%	19%
		25% Sq	S	90%	0%	2 (4)	17.6 mo NS	16%	42%
									P = 0.02
Burmeister *et al*[14], 2005	256	37% Sq	CF + R → S	80%	16%	5 (4.8)	22.2 mo	35%	15%
		62% A	S	59%	0%	6 (5.5)	19.3 mo	30%	19%
		1% mixed/ other						See text	
Tepper *et al*[15], 2008	56	25% Sq	CF + R → S	-	33%	0 (0)	4.5 yr	39%	13%
		75% A	S		0%	1 (3.8)	1.8 yr	16%	15%
							P = 0.002	5 YS	
Cao *et al*[9], 2009	366	Sq	CFM → S	87%	1.7%	0%	Approximately 42 mo	Approximately 69%	-
			R → S	98%	15%	0%	Approximately 42 mo	69%	
			CFM + R → S	98%	22%	0%	Approximately 60 mo	74%	
			S	73%	0%	0%	Approximately 42 mo	53%	
								P = 0.013	
van Hagen *et al*[16], 2012	366	23% Sq	JT + R → S	92%	29%	6 (4)	49.4 mo	58%	-
	T1-3	75% A	S	69%	0%	8 (4)	24 mo	44%	
	N0-1	2% other						*P* = 0.03	
	M0								

-: Not reported; A: Adenocarcinoma; B: Bleomycin; C: Cisplatin; F: 5-flurouracil; Gp: Group; J: Carboplatin; M: Mitomycin; MS: Median survival; NS: Non-significant; Op: Operative mortality using number of patients actually operated as denominator; pCR: Pathological complete response; R: RT; R0: No residual tumor; S: Surgery; Sq: Squamous cell carcinoma; T: Paclitaxel; V: Vinblastine; YS: Year survival.

Table 2 Pros and cons of pre-operative therapy for esophageal cancer

Pre-op therapy	Pros	Intact vascular supply allowing for potential improved oxygenation for radiotherapy
		Smaller radiotherapy volume
		Potential tumor downstaging
		Sterilization of tumor bed in preparation for surgery
		Improve resectability
	Cons	Treatment decision based on clinical stage, may over-treat patients
		Narrow window for surgical resection post CRT, may increase surgical complications with pre-op CRT
		Dysphagia and issue of nutrition support due to tumor and treatment

CRT: Chemoradiation therapy.

were not significantly different, there was a significant difference in the proportion of deaths that were due to esophageal cancer in the 2 groups (87 of 101 patients who had surgery alone *vs* 69 of 102 patients who received combined treatment CRT and surgery, $P = 0.002$). As compared with the group treated with surgery alone, the group treated preoperatively had longer disease-free survival ($P = 0.003$), a longer interval free of local disease ($P = 0.01$), and a higher frequency of curative resection ($P = 0.017$). However, there were more postoperative deaths ($P = 0.012$) in the group treated preoperatively with CRT.

In the study of Urba *et al*[13], the preoperative CRT arm had cisplatin 20 mg/m^2 per day on days 1-5 and 17-21, 5-FU 300 mg/m^2 per day on days 1-21, and vinblastine 1 mg/m^2 per day on days 1-4 and 17-20. The tumor volume was treated with 5-cm cephalo-caudad margins and 2-cm radial margins by 1.5 Gy twice daily to 45 Gy. One patient had a microscopic positive margin in the surgical specimen and received postoperative RT. This study did not give postoperative RT for patients with positive nodes, but would use it for positive margins of resection.

Burmeister *et al*[14] used 80 mg/m^2 cisplatin intravenously on day 1 followed by 800 mg/m^2 per day 5-FU given intravenously on days 1-4. RT 35 Gy/15 f per 3 wk to the midplane, was started concurrently with

chemo. The results were not statistically significant. Neither progression-free survival nor OS differed between groups [HR = 0.82 95%CI: 0.61-1.10 and 0.89 (0.67-1.19), respectively]. The CRT + S group had more complete resections with clear margins than did the surgery-alone group [103 of 128 (80%) *vs* 76 of 128 (59%), $P = 0.0002$], and had fewer positive lymph nodes [44 of 103 (43%) *vs* 69 of 103 (67%), $P = 0.003$]. Subgroup analysis showed that patients with squamous-cell tumours had better progression-free survival with chemoradiotherapy than did those with non-squamous tumours [HR = 0.47 (0.25-0.86) *vs* 1.02 (0.72-1.44)]. However, the trial was underpowered to determine the real magnitude of benefit in this subgroup.

CALGB 9781 shows the benefit of CRT before surgery despite the closure due to poor accrual[15]. Cisplatin 100 mg/m^2 and 5-FU 1000 mg/m^2 per day for 4 d on weeks 1 and 5 concurrent with RT (50.4 Gy/28 f per 5.6 wk) was followed by esophagectomy with node dissection in the trimodality arm. The median survival was 4.48 years *vs* 1.79 years in favor of trimodality therapy over surgery alone (exact stratified log-rank, $P = 0.002$).

Results from a recent multicenter phase Ⅲ randomized trial, Chemoradiotherapy for Oesophageal Cancer Followed by Surgery Study (CROSS study) showed that neoadjuvant CRT improved OS compared to surgery alone in patient with resectable (T2-3N0-1M0) esophageal or GEJ cancers[16]. Median survival was 49 mo in the neoadjuvant CRT arm and this seems to be the best median survival results achieved in the literature so far (Table 1). The CROSS study used a lower RT dose with newer chemo agents. The CRT consisted of weekly administration of carboplatin (doses titrated to achieve an area under the curve of 2 mg/mL per minute) and paclitaxel (50 mg/m^2) for 5 wk and concurrent RT (41.4 Gy/23 f per 4.6 wk), followed by surgery. The RT volume is also modest: the planning target volume (PTV) employed a proximal and distal margin of 4 cm around the gross tumor volume (GTV), and in case of tumor extension into the stomach, a distal margin of 3 cm was used. A 1.5 cm radial margin around the GTV was provided to include the area of subclinical involvement around the GTV and to allow for tumor motion and set-up variations.

Some patients may refuse to have surgery after a clinical complete response (clinCR) to preoperative CRT. From the prospective database of MD Anderson Cancer Center, 61 of the 622 trimodality-eligible patients declined surgery after a clinCR, defined as both endoscopic biopsy showing no cancer and physiologic uptake by positron emission tomography (PET)[17]. Forty-two out of the 61 patients were alive at a median follow-up of 50.9 mo (95%CI: 39.5-62.3). The 5-year overall and relapse-free survival rates were 58.1% ± 8.4% and 35.3% ± 7.6%, respectively. Of 13 patients with local recurrence during surveillance, 12 had successful salvage resection. The authors concluded that although the outcome of 61 patients with clinCR who declined surgery appears reasonable, in the absence of a validated prediction/prognosis model, surgery must be encouraged for all trimodality-eligible patients.

In 2011, Kranzfelder *et al*[18] published a meta-analysis which sought to clarify the benefits of neoadjuvant treatment: there were nine randomized controlled trials involving neoadjuvant CRT *vs* surgery, eight involving neoadjuvant chemo *vs* surgery. The HR for OS was 0.81 (95%CI: 0.70-0.95, $P = 0.008$) after neoadjuvant CRT and 0.93 (0.81-1.08, $P = 0.368$) after neoadjuvant chemo. Morbidity (HR = 1.03, $P = 0.638$) and mortality (HR = 1.04, $P = 0.810$) rates after neoadjuvant chemo and surgery did not differ from those after surgery alone. However, the 30-d mortality was non-significantly higher with combined treatment.

S vs S + RT: Postoperative adjuvant RT without chemo

Post-esophagectomy adjuvant RT can reduce local recurrence rate[19,20]. Several randomized trials were performed comparing surgery plus postoperative RT (PORT) with surgery alone to clarify the impact of PORT[21,22]. The majority of the evidence has revealed that PORT may improve local disease recurrence but does not confer any survival benefit over surgery alone[23,24]. These trials had limitations: (1) patients were not stratified by stage hence unlikely to detect an improvement in survival in those with high risk features (positive lymph nodes, deeply invading tumors); (2) they often include patients with positive celiac nodes; (3) they include mostly squamous cell carcinomas; and (4) no chemo were given. Adjuvant RT can theoretically treat microscopic disease left behind after surgery to increase local control, but cannot eradicate systemic spread of tumor cells.

Schreiber *et al*[25] performed a retrospective review using the American Surveillance Epidemiology and End Results (SEER) database to analyze whether there was survival benefit to adjuvant RT in stage T3-4N0M0 or T1-4N1M0 esophageal cancer who were definitively treated with esophagectomy. A total of 1046 patients met the selection criteria; 683 (65%) received surgery alone and 363 (34.7%) received PORT. For stage Ⅲ esophageal carcinoma (T3N1M0 or T4N0-1M0), 346 patients underwent surgery alone and 231 patients received PORT. Use of PORT resulted in an improvement in median OS from 15 to 19 mo and an improvement in 3-year OS from 18.2% to 28.9% ($P < 0.001$), respectively. This benefit was present for both squamous cell and adenocarcinoma. One limitation of the SEER data is the lack of information on use of chemo, so the benefit could be effect of CRT.

S vs S + CRT: Postoperative adjuvant CRT

Some studies[26,27] addressed the impact of PORT with chemo on node-positive esophageal carcinoma, and found a survival benefit. The randomized adjuvant CRT trial, Intergroup trial (INT 0116) enrolled 559 patients with resected adenocarcinoma of the stomach or GEJ. They were randomly assigned to surgery plus postoperative CRT or surgery alone[28]. The adjuvant arm used 425

Table 3 Pros and cons of post-operative therapy for esophageal cancer

Post-op therapy	Pros	Treatment decision based on true pathologic stage, avoid CRT in patient who may not require it
		Accurate assessment of disease extent to allow delineation of disease involvement
		Immediate relief of dysphagia due to tumor
	Cons	Difficulty to delineate RT target volume
		Large RT therapy volume and difficulty in RT planning
		Potential decrease in oxygenation to tumor bed due to postoperative tissue alteration in vascular supply
		Inability to assess RT or chemo tumor response
		May preclude the use of postoperative CRT for those patients with reduced functional status postoperatively

CRT: Chemoradiation therapy; RT: Radiotherapy.

mg/m^2 of 5-FU, plus 20 mg/m^2 of leucovorin per day, for 5 d, followed by 45 Gy/25 f per 5 wk of daily RT, with modified doses of 5-FU and leucovorin on the first 4 and the last 3 d of RT. A month after the completion of RT, two 5-d cycles of 5-FU (425 mg/m^2 per day) plus leucovorin (20 mg/m^2 per day) were given 1 mo apart. Hence a total of 4 mo cycles of adjuvant chemo was given. Twenty percent of the patients had GEJ adenocarcinoma. Subset analyses show robust adjuvant treatment benefit in most subsets.

CRT + S vs S + CRT: Preoperative vs postoperative therapy

Tables 2 and 3 compare the advantages of preoperative *vs* postoperative therapy[29,30]. There are no well performed randomized trials to compare the outcome of pre- against post-operative therapy with modern treatment staging and treatment techniques. Neoadjuvant treatments can be started immediately targeting any micro-metastatic deposits without allowing time for further cancer growth. The exact disease staging often cannot be firmly assessed at the preoperative circumstances.

Further research of the multidisciplinary management for patients with locally advanced esophageal cancer is warranted. The approach is currently being explored in two countries: China and Canada. In China the study has been carried out by investigators of the ZTOG1201 trial, a multicenter phase Ⅱ trial of neoadjuvant and adjuvant CRT in locally advanced esophageal cancer (NCT01463501)[31]. In Canada, this is undertaken by investigators of the QUINTETT phase Ⅲ trial (NCT00907543) of neoadjuvant *vs* adjuvant therapy in locally advanced esophageal cancer trial including quality of life[32]. Results of these trials can potentially provide further insight on the impact of trimodality therapy on the management of locally advanced esophageal cancers.

CRT + S vs CRT: Does surgery add to CRT?

The omission of surgery would leave residual disease behind and therefore surgery theoretically should contribute to treatment success. There were clinical trials comparing neoadjuvant CRT followed by esophagectomy to definitive CRT. Stahl *et al*[33] randomized 86 patients with advanced squamous cell carcinoma of the esophagus for neoadjuvant CRT of cisplatin, leucovorin, etoposide and 40 Gy RT followed by esophagectomy, compared to 86 patients treated with same chemo but 65 Gy RT and no surgery. The median survival was 16 and 15 mo with and without surgery, respectively. The 2-year survival rate was 40 and 35 mo with and without surgery, respectively. HR was 0.83 (0.54, 1.23) and was non-significant.

The other trial was performed by Bedenne *et al*[34]. Their trial randomized 129 patients with advanced squamous cell carcinoma of esophagus for neoadjuvant CRT of cisplatin, 5-FU, 46 Gy RT followed by esophagectomy, comparing with 130 patients treated with the same chemo but 66 Gy without surgery. The median survival was 18 and 19 mo with and without surgery, respectively. The 2-years survival was 34 and 40 mo with and without surgery, respectively. The HR was 0.88 (0.59, 1.31) and was non-significant.

In a Phase Ⅱ trial in Radiation Therapy Oncology Group (RTOG 0246)[35], definitive CRT employed induction 5-FU (650 mg/m^2 per day), cisplatin (15 mg/m^2 per day), and paclitaxel (200 mg/m^2 per day) for two cycles, followed by concurrent CRT with 50.4 Gy/28 f and daily 5-FU (300 mg/m^2 per day) with cisplatin (15 mg/m^2 per day) over the first 5 d. Salvage surgical resection was considered for patients with residual or recurrent esophageal cancer who did not have systemic disease. The study was designed to detect an improvement in 1-year survival from 60% to 77.5% (α = 0.05; power = 80%). Only 71% 1-year survival was achieved among the 43 patients enrolled from September 2003 to March 2006.

These trials had low to moderate sample size, short follow up, and the RT dose in the nonsurgical arm was above 60 Gy. This was concluded, in the meta-analysis of Kranzfelder *et al*[18] that no trials demonstrated a significant survival benefit of definitive CRT compared with neoadjuvant treatment followed by surgery, however the likelihood of R0 (no residual tumor) resection was significantly higher after neoadjuvant CRT (HR = 1.15, P = 0.043).

In the specific scenario of T4 esophageal cancers, defined as a tumor that invades neighboring structures (*e.g.*, aorta, trachea, bronchus, and lung), are usually considered inoperable despite recent advances in surgical techniques. CRT + S is superior to CRT with respect to local control and short-term survival although CRT-S is associated with relatively higher perioperative mortality and morbidity[36]. On the other hand, it is sometimes difficult to achieve local control with CRT and the treatment often

results in fistula formation, though a complete response to CRT is often associated with better prognosis. Admittedly, the difference in the survival rate between the two modalities is marginal at long-term follow-up due to operative morbidity and inadequate control of distant metastasis in CRT-S. Randomized controlled trials involving large population samples are needed to define the standard treatment for T4 esophageal cancer.

ROLE OF BRACHYTHERAPY

Esophageal brachytherapy alone is no longer used for curative situation because it can only effectively treat cancer within 1 cm radius, and unable to reach the adjacent lymphatic drainage at risk. If external beam RT is not possible, high dose rate (HDR) brachytherapy 6 Gy for 3 f or 8 Gy for 2 f at 1 cm from the center of the source axis can palliate dysphagia[37]. It should not be given concomitantly with chemo or external beam RT. The toxicity was reported by RTOG 92-07 study[38]. This phase I / II study planned to give 50 Gy/25 f per 5 wk of external beam RT followed 2 wk later by brachytherapy (either HDR 5 Gy during weeks 8, 9, and 10, for a total of 15 Gy, or low-dose-rate 20 Gy during week 8). Chemo was given during weeks 1, 5, 8, and 11, with cisplatin 75 mg/m^2 and 5-FU 1000 mg/m^2 per 24 h in a 96-h infusion. The final analysis showed severe toxicity, including treatment-related fistulas, occurred in 6/49 (12% patients, 14% among those starting brachytherapy) within 7 mo of brachytherapy.

HDR brachytherapy before external beam RT and chemo as a boost in the treatment of patients with esophageal cancer was reported to be safe in a single institution study[39]. Further investigation on the role of HDR brachytherapy boost treatment in multimodality management is needed. Other ways of brachytherapy for esophageal cancer palliation was studied, in the form of self expandable stent loaded with radioactive seeds of low dose rate brachytherapy. In a single institution small pilot study, 53 patients were randomized to an I -125 loaded stent or a conventional stent[40]. Systemic therapy was allowed for both the treatment and control group. The benefit for relief of dysphagia was significant after 2 mo ($P < 0.05$). The stent restenosis occurred later in the RT stent group than in the control group (4.75 mo *vs* 2.00 mo) ($P < 0.05$). In RT stent group, median OS was 7 mo (95%CI: 5.0-10.0) and mean OS was 8.3 mo (95%CI: 6.36-10.21). In control group, median OS was 4 mo (95%CI: 2.0-4.0) and mean OS was 3.5 mo (95%CI: 2.720-4.16) ($P < 0.001$, log-rank test).

TARGET VOLUME OF EXTERNAL BEAM RT

The ERT treatment volume for esophageal cancer is controversial. For example, distal esophageal adenocarcinomas at the GEJ may be treated with esophageal cancer RT portal instead of stomach cancer RT portal. The following section will discuss the preoperative and postoperative RT target volumes.

Preoperative and definitive RT

Tai *et al*[41] noted a great variability in target volume delineation. In the absence of a general consensus guideline, this could be due to practice variations among oncologists in individual cases. Esophageal cancer can extend submucosally in the longitudinal direction for a considerable distance. Miller *et al*[42] reported that in 15% of cases, microscopic longitudinal spread at greater than 6 cm from the primary lesion can occur. However, this cannot become the clinical tumor volume (CTV) since with expansion, the PTV would be very long cranio-caudally.

Recently lean management has been used in health care. A study from Loyola University Medical Center indicates the feasibility of applying the "plan-do-check-act" (PDCA) cycle to assess competence in the delineation of individual organs, and to identify areas for improvement[43]. With testing, guidance, and re-evaluation, contouring consistency can be obtained. The PDCA approach will ensure more accurate treatments and continual quality improvement.

In RTOG 9405, the initial target volume (50.4 Gy) encompassed 5 cm margin for the superior and inferior borders[44]. The lateral, anterior, and posterior borders of the field were 2 cm or more beyond the borders of the primary tumor. The tumor size was defined by endoscopic ultrasound (EUS), barium swallow, or computed tomography (CT) scan (whichever was larger). The primary and regional lymph nodes were included. For tumors of the cervical esophagus, the supraclavicular lymph nodes were included. A separate photon or electron boost to the supraclavicular lymph nodes was allowed to bring the total dose to 50.4 Gy. Patients randomized to the high-dose arm received a cone down of 14.4 Gy to attain a total dose of 64.8 Gy. The intent of the cone down was to treat the primary tumor only, not the regional primary lymph nodes. The superior and inferior borders of the field were decreased to 2 cm beyond the tumor. The lateral, anterior and posterior borders were the same as the initial target volume.

Image-guided RT is used in many North American Centers nowadays. The experience in MD Anderson Cancer Center showed large (> 1 cm) inter-fractional displacements in the GEJ in the superior-inferior (especially inferior) direction was not accounted for when skeletal alignment alone was used for patient positioning[45]. Because systematic displacement in the superior-inferior direction had dosimetric impact and correlated with tidal volume, better accounting for depth of breathing is needed to reduce inter-fractional variability. Patients are also advised to be nil by mouth 3 h before planning CT or daily RT so that the stomach is empty.

To summarize (Figure 1A): (1) GTV includes visible tumor on CT, barium swallow, EUS, and PET scans; (2) CTV: GTV + 1 cm radially and 3-4 cm longitudinally. One may edit for anatomic barriers: vertebral bodies, ves-

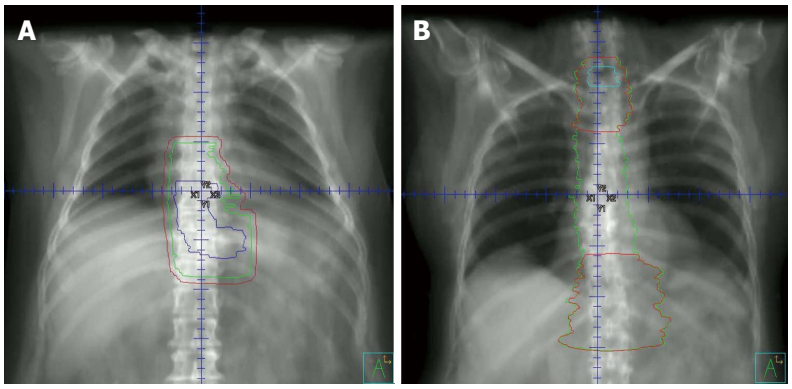

Figure 1 Radiation field for a lower esophageal cancer. A: Pre-operative with minimal involvement of gastro-intestinal junction: celiac nodes are not covered. Intensity modulated radiotherapy is used. Blue: Gross tumor volume; Green: Clinical target volume; Red: Planning target volume; B: Post- operative with involvement of gastro-esophageal junction. Intensity-modulated radiotherapy treatment. Blue: Anastomosis; Green: Clinical target volume; Orange: Clinical target volume concomitant boost, planning target volume not shown.

sels and heart. Supraclavicular nodes are covered for cervical esophagus only. Coeliac nodes are covered for lower esophageal lesions; (3) PTV: CTV + 1 cm; and (4) Field borders: generally 2 cm radial, 4-5 cm longitudinal margins. For cervical esophageal tumors, the superior field border is just below larynx. If celiac nodes to be covered, the field goes down to the bottom of T12 or L1.

Postoperative target volume

In postoperative adjuvant RT, a retrospective study of 72 high-risk patients (T3, T4, nodes positive, with or without margin involvement) treated at the London Regional Cancer Centre from 1989 to 1999 addressed the controversy whether the anastomotic site needs to be included)[46,47]. Positive/close margins were found in 34 (49%) patients. Median follow-up was 30.5 mo (range 3.4-116.3 mo). Anastomosis recurrence rates were 29% with small volume and 0% with extended volume RT (P = 0.041). Local and regional relapse occurred in 74.2% of patients treated with small volume RT compared to 15.4% in patients treated with extended volume RT (P < 0.001). After adjusting for resection margin status, the local control benefit of extended volume RT remained significant (P = 0.003).

To define the target volume, use of PET or PET/CT, alone or in combination with other methods, may be better to evaluate how far a tumour has spread (staging), whether it has responded to treatment (restaging), or detection of recurrences[48]. However, a German review of 48 studies found no strong evidence that PET, alone or in combination with CT, increases survival, improves quality of life, or results in fewer operations or diagnostic interventions[49].

To summarize (Figure 1B): (1) CTV: The tumor bed and the lymphatic drainage at risk (peri-esophageal lymph nodes and regional lymph nodes). For GEJ, the celiac nodes (around T12-L1) may need to be included; (2) PTV: CTV + 1 cm radial and longitudinal margin. The superior margin of the PTV will include the surgical anastomotic site (labeled with radio-opaque clips) proximally with 2 cm margin. The inferior margin of the field will be 5 cm beyond the previous GTV location. Lateral, anterior, and posterior borders will be 2 cm beyond the lateral borders of the tumor bed and regional lymph nodes, except if tumor bed is close to vertebral body, CTV will be on the bony surface. For the GEJ primaries, the celiac nodes (around T12-L1) may need to be included. 36-38 Gy in 28 fractions is delivered including the anastomosis. The tumor bed only should be boosted (simultaneous boost) to 50.4 Gy/28 f per 5.5 wk, together with the anastomosis if the margin is close or positive; and (3) Field borders-superiorly at about T1 to cover the anastomosis, inferiorly to L2-3 if celiac node needs to be covered.

EXTERNAL BEAM RT DOSE FRACTIONATION

Herskovic *et al*[50] (RTOG 85-01) randomized 121 patients to either 50 Gy with concurrent (75 mg/m^2) and 5-FU (1 g/m^2 per 24 h × 4 d) starting with RT for 4 cycles *vs* 64 Gy alone (Table 4). At 5 years, 27% of the combined modality patients were alive *vs* none of those in the RT alone group. For the combined modality, 27% patients had persistent disease and an additional 16% developed local recurrence, compared to 40% and 24% respectively in the RT alone group (P < 0.01). The patients who received combined treatment also had fewer distant recurrences (22% *vs* 38%, P < 0.005). A higher RT dose, 64 Gy, cannot make up for the combined benefit of CRT. However, severe and life-threatening side effects occurred in 44 percent and 20%, respectively, of the patients who received combined therapy, as compared with 25 percent and 3 percent of those treated with RT alone.

Researchers then started to investigate if high RT dose combined with chemo can further increase survival. In the Intergroup 0123 (RTOG 94-05) trial[44] the 218 eligible patients were randomized to 64.8 Gy *vs* 50.4 Gy combined with 4 mo cycles of cisplatin and 5-FU. There was no significant difference in median survival (13.0 mo *vs* 18.1 mo), 2-year survival (31% *vs* 40%), or locoregional

Table 4 Randomized trials for definitive chemoradiation therapy

Ref.	*n*	Histology	Treatment	MS	2 yr OS	Locoregional failure
Herskovic *et al*[50], 1992	121	88% Sq	CF + R 50 Gy	12.5 m	38%	43%
		12% A	R 64 Gy	8.9 m	10%	64%
					$P < 0.001$	local recurrence + persistent primary
Minsky *et al*[44], 2002	218	86% Sq	CF + R 50.4 Gy	18 m	40%	52%
		14% A	CF + R 64.8 Gy	13 m	31% (NS)	56%

A: Adenocarcinoma; C: Cisplatin; F: 5-flurouracil; MS: Median survival; NS: Non-significant; R: RT; Sq: Squamous cell carcinoma.

Table 5 Complications of radiotherapy to esophagus and their management

Acute complications
Skin erythema: 0.5% hydrocortisone, flamazine cream
Hair loss: no treatment
Mucositis, odynophagia, loss of appetite, fatigue, generalized weakness, dysphagia, dehydration, malnutrition, intestinal obstruction: intravenous hydration, xylocaine viscus, feeding tube
Pneumonitis: prednisone, oxygen
Spinal cord L'hermitte sign: no treatment
Larynx hoarseness: prednisone
Fistula/erosion of great vessels, esophageal perforation: consult thoracic surgeons
Chronic complications
Fibrosis/hyperpigmentation of skin: no treatment
Lung fibrosis: oxygen
Esophageal stricture: begins at 3-4 mo. Incidence: 50 Gy 0.8%, 60 Gy 0.6%; 60 Gy + chemo 12%. Treat by dilatation and/or stent
Peptic ulcer: proton pump inhibitor
Chronic enteritis: anti-diarrhoeal, aminosalicylates, pentoxifylline and tocopherol, cholestyramine, antibiotics, corticosteroids, hyperbaric oxygen
Spinal cord myelopathy: hyperbaric oxygen, anticoagulation

failure and locoregional persistence of disease (56% *vs* 52%) between the high-dose and standard-dose arms. Although 11 treatment-related deaths occurred in the high-dose arm compared with 2 in the standard-dose arm, 7 of the 11 deaths occurred in patients who had received 50.4 Gy or less. When comparing the high-dose arm with the low-dose arm, there was a significant prolongation of treatment time due to toxicity interruptions, and less 5-FU delivered doses.

To summarize the studies for esophageal cancer, when concurrent CRT is used without surgery, 54 Gy is recommended, although there are no firm data to support this[51]. In postoperative setting, a large elective volume (PTV1) should include the anastomosis even if the resection margins are adequate, 36-38 Gy in 28 fractions. The tumor bed should be boosted (simultaneous in field with the above mentioned PTV) to 50.4 Gy/28 f, as well as the anastomosis if the margin is close or positive[46,47]. The simultaneous integrated boost used by Yaremko *et al*[52] showed excellent result. Boost of tumor bed increases RT dose locally while a lower dose can be given to a longer clinical target volume.

COMPLICATIONS

Table 5 summarizes the acute and chronic complications for esophageal RT. To reduce complications, RT treatment modalities used in clinical research studies include 3-dimensional conformal RT (3D-CRT), intensity-modulated RT (IMRT) and proton beam therapy (PBT)[53]. When comparing the three RT modalities in 444 esophageal cancers at different locations, there was a significant increase in postoperative pulmonary complications for 3D-CRT compared to IMRT and for 3D-CRT *vs* PBT but not for IMRT compared to PBT after adjusting for pre-RT diffusion capacity of the lung for carbon monoxide (DLCO). When mean heart dose and mean lung dose (MLD) were added to multivariate analysis after adjusting for pre-RT DLCO and RT modality, the effect of RT modality was no longer significant, whereas MLD became the only significant factor for perioperative pulmonary complications.

Another study showed that IMRT compared to 3D-CRT resulted in significantly higher OS, loco-regional control, and non-cancer related mortality rates among 676 esophageal cancer patients[54].

PBT in treatment of esophageal cancer had few severe toxicities, with encouraging pathologic response and clinical outcomes[55]. It is difficult to justify PBT in esophageal cancers at the present time when there are other competing technologies available such as IMRT and until PBT facilities are more readily available as there are few centers currently in the world.

Another way to reduce complications is volumetric arc modulation. A study reported the comparison of RapidArc (RA) against 3DCRT and IMRT techniques for esophageal cancer[56]. CT scans of 10 patients were included in the study. Single-arc and double-arc RA plans were prepared to deliver 54 Gy to the PTV in 30 f. Target conformity improved with double-arc RA plans compared with IMRT. But RA plans resulted in a reduced low-level dose bath (15-20 Gy) in the range of 14%-16% compared with IMRT plans. The average monitor units needed to deliver the prescribed dose by RA technique was reduced by 20%-25% compared with IMRT technique. Therefore, volumetric arc modulation is also favored for shorter treatment time on the machine couch.

Similarly, tomotherapy significantly reduced dose to normal tissues[57]. Mean lung dose was respectively 7.4 and 11.8 Gy ($P = 0.004$) for tomotherapy and 3D plans. Corresponding values were 12.4 and 18.3 Gy ($P = 0.006$) for cardiac ventricles. Maximum spinal cord dose was respectively 31.3 and 37.4 Gy ($P < 0.007$) for tomotherapy and 3D plans.

FUTURE RESEARCH

Chemo

An important limitation of RT is its difficulty to encompass longitudinal local extension, lymphatic and nodal drainage due to normal tissue tolerance. Future research should focus on better chemo or targeted therapy to complement RT treatment. Unfortunately, epidermal growth factor receptors-targeted agents fail to improve outcomes: Panitumumab in REAL-3 trial[58] or cetuximab in SCOPE1 trial[59]. Concomitant cetuximab, cisplatin, irinotecan, and RT were poorly tolerated in the first North American cooperative group trial (S0414) testing this regimen for locally advanced esophageal cancer as treatment-related mortality approached 10%[60].

An on-going study RTOG 1010 examines the role of trastuzumab (Herceptin)[59]. Arm 1 uses RT (50.4 Gy), paclitaxel, carboplatin, and trastuzumab, followed by surgery 5-8 wk after completion of RT, then maintenance trastuzumab, every 3 wk for 13 treatments. Arm 2 does not have any trastuzumab nor any maintenance drug.

Single agent docetaxel was well tolerated in a phase Ⅱ study in China[61]. There is an on-going multicenter study on combination docetaxel, cisplatin and 5-FU in Japan[62].

A trimodal approach, consisting of a single cycle of induction chemo, CRT containing capecitabine and cisplatin, and surgery, was feasible and effective in patients with resectable esophageal squamous cell carcinoma[63]. In another study, neoadjuvant concurrent CRT with capecitabine and oxaliplatin was found to be well tolerated and effective in patients with locally advanced esophageal cancers[64].

Surgery

Improvements in perioperative management may enhance the outcome. The CRT treatment of esophageal cancer follows the example of mitomycin C and 5-FU combination in anal cancer. Recent rectal cancer research on increasing the time interval to 10-11 wk from end of neoadjuvant CRT to surgery results in the highest rate of pathological complete response for rectal cancer[65]. Similarly, future investigations of esophageal RT may pursue gradually increasing the time interval from the end of neo-adjuvant CRT to surgery to find the optimal time. Currently esophagectomy is performed 2-6 wk after completion of CRT. This will allow patients to recover from side effects of concurrent CRT by having good nutritional support prior to surgery, and to minimize any severe postoperative complications after surgery[66]. A prospective database of 266 patients in the MD Anderson Cancer Center between 2002 and 2008 showed that timing of esophagectomy after neoadjuvant CRT (within 8 wk *vs* > 8 wk) is not associated with perioperative complication, pathologic response, or OS. The authors concluded that it may be reasonable to delay esophagectomy beyond 8 wk for patients who have not yet recovered from CRT[67].

PET scan

Another area of on-going research is the use of PET scan to modify therapy. In the CALGB 80803, PET scan non-responders will cross over to the other chemo regimen[68].

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REVIEW

Endoscopic assessment and management of early esophageal adenocarcinoma

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Abstract

Esophageal carcinoma affects more than 450000 people worldwide and the incidence is rapidly increasing. In the United States and Europe, esophageal adenocarcinoma has superseded esophageal squamous cell carcinoma in its incidence. Esophageal cancer has a high mortality rates secondary to the late presentation of most patients at advanced stages. Endoscopic screening is recommended for patients with multiple risk factors for cancer in Barrett's esophagus. These risk factors include chronic gastroesophageal reflux disease, hiatal hernia, advanced age, male sex, white race, cigarette smoking, and obesity. The annual risk of esophageal cancer is approximately 0.25% for patients without dysplasia and 6% for patients with high-grade dysplasia. Twenty percent of all esophageal adenocarcinoma in the United States is early stage with disease confined to the mucosa or submucosa. The significant morbidity and mortality of esophagectomy make endoscopic treatment an attractive option. The American Gastroenterological Association recommends endoscopic eradication therapy for patients with high-grade dysplasia. Endoscopic modalities for treatment of early esophageal adenocarcinoma include endoscopic resection techniques and endoscopic ablative techniques such as radiofrequency ablation, photodynamic therapy and cryoablation. Endoscopic therapy should be precluded to patients with no evidence of lymphovascular invasion. Local tumor recurrence is low after endoscopic therapy and is predicted by poor differentiation of tumor, positive lymph node and submucosal invasion. Surgical resection should be offered to patients with deep submucosal invasion.

Key words: Esophageal adenocarcinoma; High grade dysplasia, endoscopic ultrasound; Gastroesophageal reflux; Barrett's esophagus; Chromoendoscopy; Narrow band imaging; Endoscopic mucosal resection; Radiofrequency ablation

Core tip: This review provides an up-to-date summary of the recent published studies on the use of endoscopic diagnosis and endoluminal management in patients with early esophageal adenocarcinoma, including endoscopic mucosal resection and local ablative techniques. Moreover, the review highlights the significance of this disease and the rising incidence of adenocarcinoma in the United States and western world.

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INTRODUCTION

The incidence of esophageal cancer has been increasing steadily in the United States and the western world, with a remarkable 7-fold increase in incidence in the last 30 years[1]. In fact, it has been the most rapidly increas-

ing cancer in white male population[2]. Unfortunately, the overall 5-year survival for early esophageal adenocarcinoma (EAC) has not improved and remains lower than 15%[3].

According to the National Cancer Institute (NCI), it is estimated that 17990 new cases of esophageal cancer will be diagnosed in the United States in 2013, of which approximately 60% will be adenocarcinomas[4].

The other type of esophageal cancer, esophageal squamous cell cancer continues to be the predominant type of esophageal cancer worldwide, but its incidence has been decreasing in the western countries[5]. Although genetic factors play a role in the pathogenesis of esophageal adenocarcinoma[6]. The recent dramatic increase in the incidence of esophageal adenocarcinoma is likely related to increased prevalence of gastroesophageal reflux disease (GERD)[7], increased obesity[8,9] and *Helicobacter pylori* eradication[10,11].

Reflux injury to the lower esophagus resulting in Barrett's esophagus (BE) seems to be the main precursor for EAC. This usually begins with inflammation (esophagitis), which could result after a period of time into intestinal metaplasia (BE) with increased risk to progress to dysplasia and eventually EAC[12]. In addition to acid reflux, bile acid reflux may also play an important role in the progression from Barrett esophagus to esophageal adenocarcinoma. Bile acids are synthesized from cholesterol and down-regulate caveolin-1 in esophageal epithelial cells through sterol responsive element-binding protein[13]. Caveolin-1 protects squamous epithelial cells. Moreover, bile acids increase reactive oxygen species production and cell proliferation *via* activation of PI-PLCgamma2, ERK2 MAP kinase, and NADPH oxidase NOX5-S, thereby contributing to the development of esophageal adenocarcinoma[14].

BE is two to three times more common in men than in women, and is more common in Caucasians. It is less common in African American and is extremely uncommon in Asians[15]. The risk of progression to adenocarcinoma in nondysplastic BE appears to be small. A recent population based study from the Denmark that followed 11028 patients with BE for a median of 5 years reported an annual risk of EAC of 0.12%[16].

The risk of progression to cancer increases in the presence of dysplasia and is up to 6% in patients with high grade dysplasia (HGD)[17].

Risk factors for progression of BE into cancer include low grade dysplasia (LGD), abnormal DNA ploidy and certain lectin binding patterns. Other biomarkers for progression include aberrant DNA methylation changes, expression of microRNAs, as well as overexpression or loss of expression of p53[18].

Endoscopic therapy with curative intent can only be undertaken when the risk of lymph node metastasis is negligible. It is estimated that the rate of lymph node spread is 0% in case of HGD and 1%-2% in case of intramucosal cancers (IMCs). The rate increases to 22% in case of submucosal invasion[19,20].

Table 1 Paris classification of superficial lesions

Type	Lesion
0-Ⅰ	Protruding/polypoid
0-Ⅰp	Pedunculated
0-Ⅰs	Sessile
0-Ⅱ	Non-protruding/non-excavated
0-Ⅱa	Slightly elevated
0-Ⅱb	Flat
0-Ⅱc	Slightly depressed
0-Ⅲ	Excavated

Protruding (0-Ⅰ), depressed (0-Ⅱc) and excavated (0-Ⅲ) lesions have been identified as carrying a higher risk of submucosal invasion[118].

ENDOSCOPIC DIAGNOSIS OF BE AND EARLY EAC

The diagnosis of BE is usually suspected on forward viewing upper endoscopy and is confirmed with histologic examination. Careful examination by high-resolution forward-viewing white-light endoscopy is recommended[21,22]. In a study by Gupta *et al*[23] post hoc analysis of an enriched study population and experienced endoscopists at tertiary referral centers. The authors showed that Longer time spent inspecting the BE segment (BIT) is associated with increased detection of HGD/EAC. Endoscopists who had an average BIT > 1 min per centimeter of BE detected more endoscopically suspicious lesions. Multiple random biopsies should be obtained from the four quadrants every 2 cm in non-dysplastic BE segments and every 1 cm if there is suspicion or history of dysplasia (Seattle protocol). Any visible nodule or lesion is usually suspicious for dysplasia or malignancy and should be sampled separately. Accurate description of the location, size and endoscopic appearance of the lesion is necessary for planning future therapy. Endoscopic description of lesions is usually done using the Paris classification of superficial neoplastic lesions (Table 1), which can help predict submucosal invasion in the digestive tract[24].

When confirmed histologically, the current standard of care for BE surveillance involves careful inspection using high resolution white light endoscopy with random biopsies of the BE segment according to the Seattle protocol and targeted biopsies of any suspicious areas. Multiple studies have shown that the random biopsy protocol has low sensitivity for the detection of early neoplastic changes in BE and has low adherence among endoscopists (50%)[25,26]. Furthermore, a cost-utility analysis by Gordon *et al*[27] concluded that the endoscopic surveillance of patients with non-dysplastic BE is unlikely to be cost-effective for the majority of patients and depends heavily on progression rates between dysplasia grades unless new technologies improve the quality adjusted survival benefit from the surveillance[27].

Resorting to a "random" biopsy protocol reflects the difficulty to recognize early neoplastic changes in BE. One of the reasons for this is the fact that flat lesions (such as Paris 0-Ⅱa and 0-Ⅱb lesions, Table 1) are by far

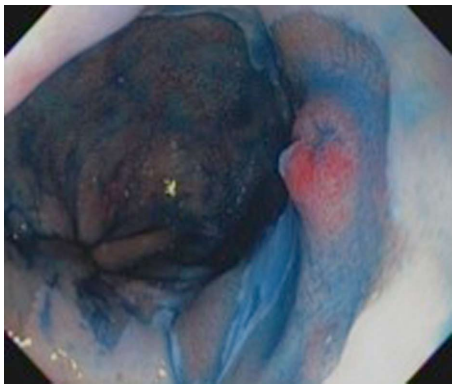

Figure 1 Chromoendoscopy with indigo carmine showing dysplastic nodule in a background of Barrett's mucosa.

the most frequent macroscopic type of neoplastic lesion in BE, and these lesions are typically hard to detect using the standard while light endoscopy[28].

Therefore, there has been major development in image enhancement techniques to improve the detection of early neoplastic lesions in BE. These techniques include detection techniques "red flag" that cover a wide area and help detect a suspicious lesion, and characterization techniques that provide detailed information about a specific area.

DETECTION TECHNIQUES

Dye-based chromoendoscopy

Dye-based Chromoendoscopy consists of spraying the Barrett's mucosa with a dye to better evaluate the microarchitecture of the mucosa to detect early neoplastic changes. Methylene blue was used in the past for this purpose[29-31]; however, it had largely fallen out of favor due to many reasons including difficulty of use and concerns on mutagenesis[32,33]. Indigo carmine is a contrast stain that permeates into the mucosal surface pits and crevices which helps to accentuate any mucosal irregularities[34] (Figure 1). Since it is not absorbed by cells, it does not have safety concerns like methylene blue. A study of 80 patients with suspected BE using high magnification chromoendoscopy with indigo carmine. The yield of intestinal metaplasia (IM) on target biopsies was 97% and 100% for HGD. However, it was not able to distinguish LGD from non-dysplastic intestinal metaplasia[35].

Acetic acid has also been used and provides magnified aspect of the mucosal architecture to help differentiate neoplastic tissue[36]. Curvers *et al*[37] demonstrated that the addition of indigo carmine and acetic acid didn't actually improve the diagnostic yield for early neoplastic lesions in BE compared to high resolution white light endoscopy. Dye-based chromoendoscopy can be labor intensive and is operator dependent and may prolong the procedure. Moreover, it has not been shown to consistently improve the detection of early neoplasia in BE.

Virtual (Dye-less) chromoendoscopy

This includes narrow band imaging (NBI) which uses optical filter to limit the white light illumination to narrow bands of light wavelengths (blue and green), which is predominantly absorbed by hemoglobin and can highlight the capillary network. This results in enhancement of the mucosal vascular and pit patterns and allows visualization of any subtle mucosal irregularities and alteration in vascular patterns concerning for early neoplastic changes[38]. Using pooled data from five studies, Curvers *et al*[39] showed promising results with NBI for detection of early neoplasia in BE with sensitivity of 97%, specificity of 94% and overall diagnostic accuracy of 96%. However, other studies showed a much lower accuracy (71%)[40].

Other virtual chromoendoscopy techniques include Pentax i-Scan and Fujinon intelligent color enhancement. These techniques use post-acquisition image computer reconstruction to enhance mucosal and vascular patterns.

At this time, there is little evidence that chromoendoscopy techniques (both dye-based and dye-less) provide improvements in the characterization and detection of early neoplasia in BE.

Autofluorescence imaging

This technique uses fluorescence radiation following excitation of tissue using light of short wavelengths, which allows differentiation of neoplastic and normal tissue. Autofluorescence imaging (AFI) has been shown to significantly improve the detection of neoplasia in BE; however, the false positive rate is very high (up to 80%)[41]. AFI has also been studied in combination with high resolution endoscopy and NBI, so called Endoscopic Trimodal Imaging (ETMI). In a multicenter randomized trial, ETMI improved the targeted detection of early neoplastic lesions compared to standard video endoscopy. However, the overall histologic yield was not different[42].

Optical coherence tomography and volumetric laser endomicroscopy

Optical coherence tomography produces high quality cross-sectional images of the mucosa based on measuring the rate of backscattering of near-infrared light. This is usually achieved using a probe that is passed through the operative channel of the endoscope. Evans *et al*[43] developed a scoring system for optical coherence tomography (OCT) and reported a sensitivity of 83% and specificity of 75% in the detection of early neoplasia in BE.

Volumetric laser endomicroscopy, the second generation from of OCT, was shown to image the esophageal mucosa at a higher speed and obtain a better quality images[44]. The recent improvements in OCT technology make it a promising technique that can achieve the goal of wide field scanning (detection) as well as characterization of a specific area of concern.

CHARACTERIZATION TECHNIQUES

Endoscopic ultrasound

Endoscopic ultrasound (EUS) may play a little role in the evaluation of patients with HGD or early adenocarci-

noma and is not routinely recommended for evaluation of flat BE segments with HGD[45,46]. A systematic review by Young *et al*[47] showed that the diagnostic accuracy for EUS staging in early EAC was only 65%. A subsequent larger meta-analysis showed better accuracy for EUS in staging T1a and T1b lesions with the area under a receiver operating characteristic curve ≥ 0.93[48]. The use of high-frequency ultrasound catheter probe (miniprobe) can provide a significant better T staging than conventional radial EUS; however, the accuracy is low with both techniques (64% and 49% respectively)[49]. Nevertheless, the National Cancer Comprehensive Network recommends EUS staging prior to proceeding with mucosal resection in the setting of esophageal carcinoma.

Confocal laser endomicroscopy

This is an imaging technique that obtains real-time 1000-fold magnified view of the mucosa, and provides histological information of the target areas (so called virtual histology). Confocal laser endomicroscopy (CLE) could be performed using a dedicated CLE endoscope or miniprobes that can be used with regular large working channel endoscopes (probe-based CLE). A recent study showed that a combination of CLE and while light endoscopy increased the sensitivity for detection of early neoplastic changes compared to white light endoscopy (76% *vs* 34%)[50]. Disadvantages to this technique include that it is expensive, time consuming and requires intensive training.

Spectroscopy

This technique relies on the principle of light interaction with esophageal mucosa to generate a biochemical profile that reflects the cellular architecture. Early results appear to be promising for the real-time detection and diagnosis of esophageal adenocarcinoma with an accuracy of 96%[51]. More recently, Almond *et al*[52] used a novel probe-based endoscopic Raman spectroscopy in *ex vivo* esophageal tissue samples and showed sensitivity of 86% and specificity of 88% for detecting early neoplasia in BE.

The above mentioned enhanced imaging techniques are not widely used in clinical practice due to the limited diagnostic accuracy, high inter-observer variability and high cost. It is also unlikely that these techniques will replace standard high resolution white light endoscopy and random biopsies for surveillance in BE; however, they could play an important role in further characterization and grading of suspicious lesions detected during surveillance exams.

Histopathologic diagnosis

Neoplastic changes in BE can be classified as LGD, HGD, *in situ* (or intraepithelial) carcinoma, IMC and invasive carcinoma[53].

Mucosal lesions are further divided into M1 lesions (or *in situ* carcinoma) when the lesion is limited to the epithelial layer, M2 lesions when the lesion invades the lamina propria and M3 when the lesion invades into but not through the muscularis mucosa layer. Lesions that invade into the submucosal are labeled SM lesions. SM lesion can be further divided into SM1 lesions when the lesion invades into the upper one third of the submucosal, SM2 lesions when the lesion invades the middle third and SM3 lesions when the lesion invades the deep one third of the submucosal layer[54].

Pathologists should carefully evaluate biopsy or resection specimens of esophageal neoplasms to provide details about tumor depth of invasion, tumor differentiation (well, moderate and poorly differentiated), lymphovascular invasion and the presence of tumor invasion at the resection margin. Lymphovascular invasion and poorly differentiated histology increases the risk of lymph node metastasis and these patients should ideally be referred for surgical resection[55].

HGD

HGD is characterized by marked cytological atypia and distorted architecture. Architectural distortion changes include marked crypt crowding, crypt budding and branching. Cytologically, HGD shows cells with marked nuclear pleomorphism, increased N/C ratio, and an increased number of atypical mitoses, particularly in the upper levels of the crypts. Goblet and Paneth cells are usually scarce or absent. Adenomatous (intestinal) dysplasia is the most common subtype but non-adenomatous (foveolar) dysplasia has also been described[56].

Immunohistochemistry staining could help in the diagnosis of HGD. Promising markers include p53 and α-methylacyl coenzyme A racemase but these are not widely used yet[57,58]. Given the significant intraobserver and interobserver variability in the diagnosis of LGD and HGD in BE, most gastrointestinal (GI) societies recommend that a second experienced gastrointestinal pathologist confirm the diagnosis[59]. It is noteworthy that the Japanese and some European pathologists don't use the term HGD and prefer to use the term *in situ* carcinoma for these lesions[60].

Intramucosal carcinoma

IMC invades through the basement membrane to the lamina propria and the muscularis mucosa. It is characterized by atypical cells or complex glands invading into the lamina propria. It is extremely important to differentiate between IMC (or T1a lesion) and carcinoma invading into the submucosa (T1b) as the distinction carries significant implications for the risk of lymph node metastasis and therapy. Such distinction is often difficult to make on biopsy specimens and larger resection specimens such as that resulting from endoscopic mucosal resection (EMR) are more helpful to distinguish between T1a and T1b lesions. In one study, 45% of patients had their final pathological stage changed after EMR compared to pre-EMR forceps biopsies[61]. It also known that most BE usually has double muscularis mucosa layer but this has no impact on the classification or the treatment of Barrett's adenocarcinoma[62].

STAGING OF EARLY ESOPHAGEAL ADENOCARCINOMA

Several modalities have been used to stage esophageal adenocarcinoma. These include EUS, endoscopic mucosal resection with histological assessment and computed tomography/positron emission tomography (CT/PET). EUS and EMR are currently applied as staging tools for early esophageal adenocarcinoma. Early cancer is defined as T1sm1, as beyond this point metastases increases from 1% to 10% for T1sm2 based on a recent consensus[63]. Stage T1a malignancies include lesions confined to the mucosa: M1 (intraepithelial), M2 (lamina propria invasion), or M3 (muscularis mucosa invasion). Submucosal or T1b malignancies are classified into Sm1 (superficial submucosa invasion), Sm2 (invasion to center of submucosa), or Sm3 (invasion to deep submucosa). Mucosal (T1a) malignancies have extremely low risk of local lymph node progression while submucosal invasion (T1b) markedly increases the risk of lymph node metastases[64,65].

EUS

The clinical utility of EUS for staging patients with BE and high-grade dysplasia or intramucosal carcinoma prior to endoscopic therapy has a limited accuracy. The principal role of EUS in evaluating patients with Barrett's-associated dysplasia is to identify patients who may be candidates for endoscopic ablative therapy such as endoscopic mucosal resection and/or photodynamic therapy. EUS has been shown to be superior to computed tomography or magnetic resonance imaging for preoperative staging in patients with high-grade dysplasia and carcinoma. EUS is considered the best tool for T and N staging of esophageal cancer, however, its performance in early Barrett's neoplasia is suboptimal for tumor depth assessment. In a meta-analysis by Puli *et al*[66] the pooled sensitivity of EUS in T1 disease was (88.1%), T2 (82.3%), T3 (89.7%) and T4 (99.2%). EUS can identify nodal spread (N1) or deep tumor invasion (T3) for which it precludes surgical resection. The risk of nodal involvement in early esophageal cancer confined to the mucosa (T1a) ranges between 0% and 3%, and when the lesion extends into the submucosal layer (T1b) this risk approaches up to 30%-50%[67-69]. Tumor size (OR = 1.35 per centimeter, 95%CI: 1.07-1.71) and lymphovascular invasion (OR = 7.50, 95%CI: 3.30-17.07) were the strongest independent predictors of lymph node metastasis[70]. In a retrospective analysis of 135 with HGD (79%) or IMC (21%) who had staging by EUS. Pathologic lymph nodes or metastases were not found by EUS. There were no endosonographic abnormalities noted in any patient with non-nodular mucosa (0/79). However, abnormal EUS findings were present in 14% with nodular neoplasia (five IMC, three HGD)[71]. For patients with nodular neoplasia, endoscopic mucosal resection of the nodule with histological examination had greater utility than staging by EUS. The use of high frequency ultrasound catheter probe (HFP) have been studied in two large studies included 94 and 106 subjects[72,73]. Both studies revealed that HFP is significantly better for lesions localized in the tubular esophagus than the gastroesophageal junction. Moreover, the performance of HFP in assessing submucosal involvement is poor. At this time EUS and HFP staging technique is inadequate for predicting T1-2N0 disease in esophageal adenocarcinoma[74].

Endoscopic mucosal resection

Endoscopic mucosa resection (EMR) has taken a central role in the staging and treatment modality for patients with early esophageal adenocarcinoma, as it allows the pathologist to provide tumor-staging information necessary for an appropriate clinical management decision process. In fact, it is the most accurate staging procedure to assess depth of invasion if full submucosa is provided in the specimen. By providing full thickness of the resected submucosa, pathologists are able to provide a clear histologic depth of the tumor (T staging) and evaluate for lymphovascular invasion. EMR provides better staging for visible lesions than do biopsies alone. Moreover, endoscopic mucosal resection may result in changing the histologic diagnosis in patients with BE with visible and flat neoplasia. In a multicenter study which evaluated 138 patients with BE-related neoplasia who undergone endoscopic eradication therapy showed EMR resulted in a change of the histologic diagnosis in 31.1% patients (upgrades 10.1%; downgrade 21%) with or without visible lesions[75]. At this time, EMR appears to be superior to biopsy for diagnosing and staging superficial esophageal tumors and can substantially modify the diagnostic grade of a lesion. Therefore EMR may facilitate optimal therapeutic decisions by avoiding undertreatment and overtreatment based on inaccurate grading and staging[76].

CT/PET

Early use of PET in the staging of patients with esophageal cancer could facilitate treatment planning and identifying unsuspected distant metastases in up to 20% of patients with a negative metastatic survey by conventional staging[77]. Positron emission tomography detects more distant lymph node and organ metastases compared with conventional diagnostics, allowing a more accurate selection of the most appropriate treatment. CT/PET has inadequate assessment in the superficial esophageal adenocarcinoma. Moreover, the addition of PET to a complete EUS examination did not alter regional-node or celiac-node staging in patients with esophageal cancer[78]. SUVmax ratio was only associated with tumor invasion depth on CT/PET. A recent study evaluated the use of CT/PET in early esophageal adenocarcinoma using a cut-off of 1.48, the sensitivity and specificity of SUVmax ratio for identification of T1a lesions were 43.3% and 80.9%, respectively[79]. Thus more data is needed on the role of CT/PET in early EAC.

ENDOSCOPIC MANAGEMENT OF EARLY ESOPHAGEAL ADENOCARCINOMA

The management of patients with early esophageal cancer

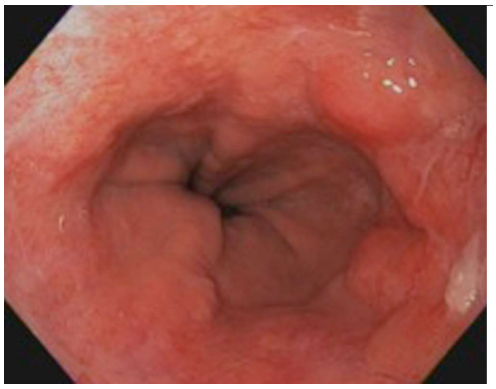

Figure 2 Barrett's esophagus with nodularity concerning for dysplasia or malignancy between 1 and 5 o'clock.

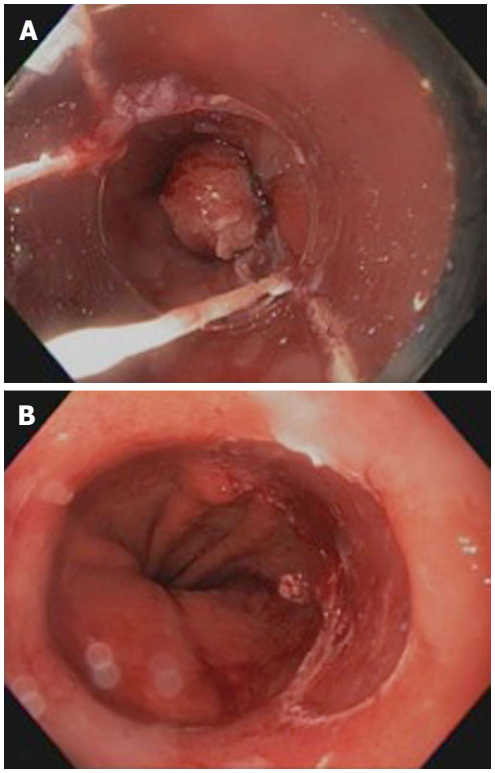

Figure 3 Endoscopic mucosal resection. A: Using Band ligation of Barrette's esophagus nodule; B: Defect after endoscopic mucosal resection using band ligation and resection of Barrett's esophagus nodules.

considered for treatment should take place in a specialty multidisciplinary team including GI pathologist, esophageal surgeon, therapeutic endoscopist, radiologist and oncologist. The endoscopic treatment should commence in high volume tertiary referral centers with availability and expertise in the multiple modalities of endoscopic therapy of BE. Moreover, the center must possess expertise in the management of complications of each modality. The British Society of Gastroenterology recommended a minimum of 30 supervised cases of endoscopic resection and 30 cases of endoscopic ablation should be performed to acquire competence in technical skills, management pathways and complications. Patients with EAC should be informed about the benefits, risks and alternatives of endoscopic and surgical approach. Initially, endoscopic mapping of the Barrett's segment with intestinal metaplasia should be undertaken prior to any endoscopic therapy. The American Gastroenterological Association (AGA) recommends endoscopic eradication therapy for patients with high-grade dysplasia. Risk stratification based on histopathologic assessment should be performed and any nodularity seen on white-light forward viewing upper endoscopy should undergo resection prior to any local ablative therapy (Figure 1). Lymph node metastasis should be excluded. Endoscopic therapy appears to be a good alternative to esophagectomy for patients with low risk pT1b sm1 EAC, on the basis of macroscopic and histologic analyses[55,80]. Data obtained from the Surveillance Epidemiology and End Results database of the NCI to compare cancer-free survival in patients with early esophageal cancer who were either treated with endoscopic therapy (n = 99) or surgical resection (n = 643) did not reveal a difference in esophageal cancer-specific mortality between the two groups[81]. In a population-based analysis, the use of endoscopic therapy for superficial EAC tended to increase from 1998-2009 and the long-term survival of patients with EAC did not appear to differ between those who received endoscopic therapy and those treated with surgery[82]. Several curative modalities are available for local treatment of BE with HGD. Among these modalities are radiofrequency ablation, argon plasma coagulation, thermal laser therapy, cryotherapy and photodynamic treatment. Here we review the efficacy and risks of each modality. Long term outcome of patients with BE and HGD who underwent endoluminal therapy revealed recurrence of intestinal metaplasia occurs in one-third of cases and supports continued endoscopic surveillance even after complete eradication[83].

EMR

Endoscopic mucosal resection provided a primary role in the endoscopic therapy of patients with early EAC (HGD, T1a). EMR should not be attempted if lymph node invasion is suspected. EMR should be performed by an expert therapeutic endoscopist. The principle of EMR is to capture the entire mucosa and submucosa using a suction cup fitted on the tip of the endoscope (Cap-assisted suck and cut or band and cut technique) or lifting the submucosa from the muscularis propria through submucosal injection of saline or indigo carmine (freehand technique). The entire specimen is then excised *en bloc* using a diathermy snare resection or performing multiband mucosectomy[84] (Figures 2 and 3). Total *en bloc* resection is preferred to reduce risk of recurrence and provide accurate histologic assessment. The distinct advantage of EMR over ablative therapy is providing large specimen of resected tissue for histopathologic assessment. One must understand the limitations of EMR include the assessment of base and lateral margin of the tumor resected specimen. The depth of infiltration is better assessed using quantitative micrometric measure in microns

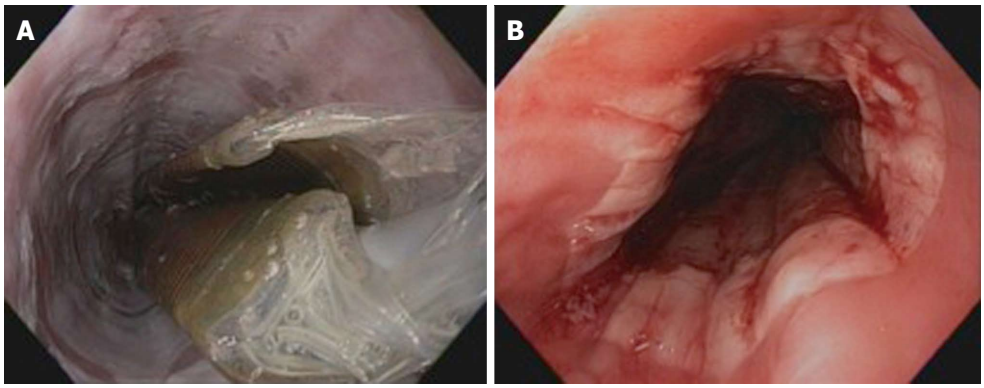

Figure 4 Barrett's esophagus. A: Ablation of Barrett's esophagus using the circumferential balloon catheter; B: Barrett's esophagus after the first round of ablation using the circumferential balloon ablation catheter.

of the depth of submucosal invasion from the bottom of muscularis mucosae. This is deemed to be more accurate than classifying tumor invasion based on depth of submucosal involvement (sm1, sm2, and sm3) as the entire submucosa may not be available in the specimen of all cases[85]. EMR can also be performed in patients with early esophageal adenocarcinoma with previous antireflux surgery[86]. Risk of recurrence after EMR appears low. In one study evaluating 22 patients (16 with HGD), 82% had no evidence of HGD or cancer after a median follow-up of two years[87]. Another long-term follow up study carried in 7 patients for more than 10 years, in 43 for 5-10 years, in 31 for 3-5 years and in 66 for less than 3 years after endoscopic resection. Of the 11 patients who died during the follow up, 10 died of other diseases, only 1 of recurrence of tumor. The 5-year survival rate was 96.2% for early-stage esophageal cancer[88]. Risks of EMR include bleeding, perforation and stricture formation which can occur in up to 37% of cases[61].

Endoscopic submucosal dissection

Endoscopic submucosal dissection is an advanced endoscopic procedure to resect early gastrointestinal neoplasms. It is technically more difficult, carries a high risk when used to treat early esophageal tumors and currently is not widely available in the United States. Studies have been published and reported its efficacy and safety in patients with early EAC[86,89]. In a phase Ⅱ study of endoscopic submucosal dissection for superficial esophageal neoplasms to assess the efficacy and safety of endoscopic submucosal dissection (ESD) in 56 lesions, the *en bloc* resection rate and R0 resection rate were 100% and 94.6%, respectively. The median treatment time for completing the procedure was 69 min (24-168 min)[90]. The rates of adverse events during and after ESD were 22.2% and 53.8%, respectively, but most events were mild. Another study evaluated ESD in combination with radiofrequency ablation in 30 patients with biopsy-proven mucosal adenocarcinoma. Endoscopic follow-up (median 17 mo) showed complete remission of neoplasia in 27/28 (96.4%) patients who underwent successful ESD using waterjet-assisted system[90]. A Meta-analysis by Cao *et al*[91] of endoscopic submucosal dissection *vs* endoscopic mucosal resection for tumors of the gastrointestinal tract showed higher *en bloc* and curative resection rates (OR = 13.87, 95%CI: 10.12-18.99; OR = 3.53, 95%CI: 2.57-4.84) irrespective of lesion size. Subgroup analysis showed higher *en bloc* and curative resection rates with ESD for esophageal, gastric, and colorectal neoplasms, and for lesions of size < 10 mm, 10 mm < 20 mm, and > 20 mm and lower local recurrence. However, ESD was more time-consuming than EMR and showed high procedure-related bleeding and perforation rates (OR = 2.20, 95%CI: 1.58-3.07; OR = 4.09, 95%CI: 2.47-6.80). Similarly, in a previous study evaluating the role of ESD in comparison to EMR in 171 lesions ≤ 20 mm of esophageal cancer (168 were squamous-cell carcinoma and 3 were adenocarcinoma), the curative resection rate of ESD was 97% significantly higher than endoscopic mucosal resection cap-assisted (87%)[92]. However, EMR would be an alternative to lesions < 15 mm in diameter. One must note that ESD in the esophagus has been associated with perforation rates of 2% to 5% and stricture rates between 5% and 17.2%[90,93]. More data is needed to evaluate the utility of ESD for early esophageal adenocarcinoma in the United Stated.

Radiofrequency ablation

Radiofrequency ablation of BE with HGD is the most commonly used therapy, which has been shown to produce reproducible superficial injury in the esophagus (Figure 4). Its ease of use and better safety profile makes it a favorable therapy for flat lesions with HGD. The system generator is capable of delivering 10 to 12 J at a setting of 40 W/cm² with a depth of ablation between 500 and 1000 mm. Two delivery systems are currently available in use. A 3-cm-long balloon ablation catheter (HALO 360) intended to treat long-segment circumferential BE, and an endoscope-mounted targeted device (HALO 90) to treat short segments and tongues of BE. In a recent large series of 335 patients with BE and neoplasia (72% with HGD, 24% with IMC, 4% with low-grade dysplasia) in the United Kingdom who underwent RFA for BE-related neoplasia. The authors found that by 12 mo after

treatment, dysplasia was cleared from 81% of patients. Shorter segments of BE respond better to radiofrequency ablation (RFA)[94]. In another study of 70 patients who were treated. Seventy-four per cent had dysplasia (44 LGD, 8 HGD). Complete response was accomplished in 81% of patients[95]. A United Kingdom registry that follows the outcomes of 335 patients with BE who have undergone RFA for neoplasia and received endoscopic mucosal resection if nodules are found revealed HGD was cleared from 86% of patients, all dysplasia from 81%, and BE from 62% at the 12-mo time point, after a mean of 2.5 (range, 2-6) RFA procedures[94]. Of interest, endoscopic mucosal resection before RFA did not provide any benefit. Moreover, RFA appears to have a higher rate of complete histologic resolution response in comparison to photodynamic therapy (PDT) without any serious adverse events and was less costly than PDT for endoscopic treatment of Barrett's dysplasia[96]. Complications of RFA include chest and cervical pain, abdominal pain, dysphagia and stricture formation. Subsquamous neoplasia have been reported to develop after RFA for BE[97]. Currently, RFA is reserved for patients with BE with high-grade dysplasia with no visualized nodules. Its application for patients without dysplasia is debatable giving risks of complications and cost[98].

Photodynamic therapy

Photodynamic therapy has been used to photochemically eliminate abnormal mucosa. Porfimer sodium (POR) PDT use has been limited by serious side effects including prolonged cutaneous photosensitivity and stricture formation. In a randomized phase Ⅲ trial using POR and photodynamic therapy for ablating HGD in conjunction with omeprazole, POR PDT appears to be an effective therapy for ablating HGD in patients with BE and in reducing the incidence of esophageal adenocarcinoma[99]. PDT is associated with increased risks of stricture formation and of buried intestinal metaplasia or malignancy underneath neosquamous epithelium. In a study by Weiss *et al*[100] on 17 patients treated with PDT. High-grade dysplasia or early adenocarcinoma was completely eliminated in nine of 60% patients. Complications included stricture, sunburn, urticaria, small pleural effusions, esophageal spasm and transient atrial fibrillation. A recent randomized controlled trial of 5-Aminolaevulinic acid (ALA) *vs* Photofrin photodynamic therapy for high-grade dysplasia arising in BE showed no difference in complete reversal of HGD between the two groups. On sub-group analysis for BE ≤ 6 cm, complete reversal of HGD was significantly higher with ALA-PDT than Photofrin-PDT. Strictures and skin photosensitivity were significantly more common after treatment with Photofrin-PDT than ALA-PDT (33% *vs* 9% and 43% *vs* 6%, respectively, $P < 0.05$)[101].

Argon plasma coagulation

Argon plasma coagulation is a noncontact thermal tissue coagulation in which argon gas provides the medium for the delivery of an electric current[102]. This is accomplished with passing a probe through the working channel of the endoscope. The general setting for ablation of Barrett's mucosa is a high power setting 60-90 W at 1-2 L/min. Earlier study showed complete eradication of HGD and *in situ* adenocarcinoma was achieved after a mean number of 3.3+/-1.5 V. Argon plasma coagulation (APC) sessions in (80%)[103]. In a randomized controlled trial of 35 patients who received ablation of BE with multipolar electrocoagulation (16) *vs* argon plasma coagulation (19), the authors concluded complete reversal of BE can be maintained in approximately 70% of patients, irrespective of the technique[104]. Similarly, previous studies showed similar outcome with eradication of BE and restoration of squamous epithelium[105]. However, progression to HGD can still occur despite APC ablation[106]. Thus APC is effective ablative therapy for BE but the long term benefits are unknown. More data is needed on its use in early EAC.

Cryotherapy

Cryoablation is a relatively new technique with studies focusing on high-grade dysplasia and early-stage cancer in high-risk patients. It has an acceptable safety profile, and early results show response in a significant number of patients in whom other modalities have failed[107]. Its ease of use and lower chance of complication make it an attractive procedure. Although cryoablation is a non-tissue acquiring procedure that requires liquid nitrogen spray application it is not devoid of potential risk of gastric perforation due to gas insufflation. Data on its use in early EAC is limited. In a multicenter, retrospective cohort study of 79 patients with esophageal carcinoma in whom conventional therapy failed, refused and/or were ineligible for conventional therapy[108]. The study included all T staging and showed complete response of intraluminal disease in 31 of 49 subjects (61.2%), including 18 of 24 (75%) with mucosal cancer with an overall follow up of 10.6 months. No serious adverse events were reported. A recent study by Gosain *et al*[109] evaluated 32 patients with BE-HGD of any length who were treated with liquid nitrogen spray cryotherapy every 8 wk until complete eradication of HGD and intestinal metaplasia. Complete eradication of HGD achieved in 100% (32/32), and IM in 84% at 2-year follow-up. Recurrent HGD occurred in 18% with HGD. BE segment length ≥ 3 cm was associated with a higher recurrence of IM but not HGD. No serious adverse events occurred although stricture was seen in 9% of cases. Thus, cryoablation therapy appears comparable to other treating modality in BE and in early EAC, spray cryotherapy appears to have a unique role, eliminating mucosal cancer in 75% of patients[110].

A recent meta-analysis of seven studies involving 870 patients who underwent endotherapy (n = 510) or surgery (n = 360) concluded that endotherapy has similar efficacy to surgery but with lower adverse event rates. However, endotherapy was associated with a higher neoplasia recurrence rate[111]. Limitation to this study included small number of retrospective studies and different types

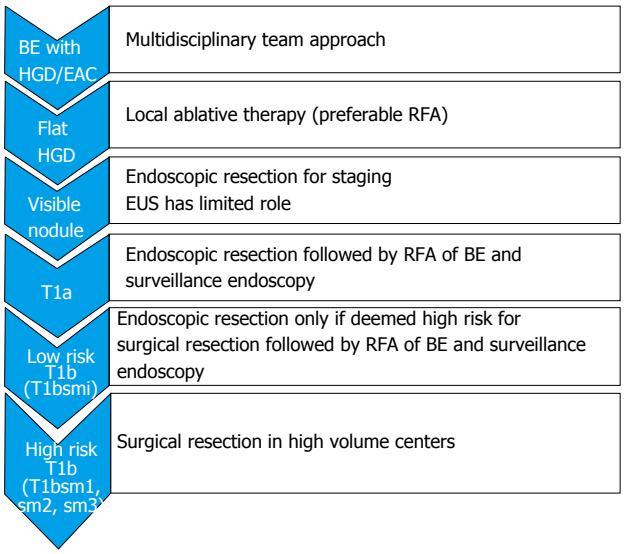

Figure 5 The current practical approach for patients with early esophageal neoplasia. BE: Barrett's esophagus; HGD: High grade dysplasia; EAC: Esophageal adenocarcinoma; EUS: Endoscopic ultrasound.

of endoscopic treatments used. Figure 5 shows the current practical approach to the management of patients with early EAC.

ROLE OF CHEMOPREVENTION

Esophageal adenocarcinoma is characterized by increasing incidence, male predominance and lack of preventive measures. Future preventive therapy might include the treatment of gastroesophageal acid reflux, obesity and/or chemoprevention with nonsteroidal antiinflammatory (NSAIDs) drugs or statins. Today, there is no evidence-based preventive measures are currently available for patients with EAC. Proton pump inhibitors are effective in reducing esophageal acid exposure and improve reflux symptoms however, they are not recommended for use as chemopreventive agents in EAC. Weight loss, exercise and bariatric surgery may potentially improve obesity. Studies have shown up-regulation of cyclooxygenase (COX)-2 in BE-metaplastic and dysplastic tissue and in Barrett's adenocarcinoma[112-114]. Others showed conflicting results[115]. NSAIDs and COX inhibitors have been proposed and shown to reduce risk of metaplasia in BE and EAC[116]. Statins have been suggested to induce anticancer effects against a variety of cancers in several studies[117]. Agents targeting the vascular endothelial growth factor and epidermal growth factor receptor pathways are currently in progress. The AGA recommendation for the chemoprevention of cancer in patients with BE is screening patients to identify cardiovascular risk factors for which aspirin therapy is indicated and against the use of aspirin solely to prevent esophageal adenocarcinoma in the absence of other indications[22].

CONCLUSION

Esophageal cancer is one of the most serious gastrointestinal cancers worldwide, owing to its rapid development and fatal prognoses in most cases. Major risk factors for EAC include BE, GERD, smoking, and obesity. Improved survival is achievable when the disease is confined to the more superficial mucosal layers and treated. Endoscopic luminal therapy is feasible and proven useful in BE with HGD and early esophageal adenocarcinoma.

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ORIGINAL ARTICLE

In vitro effects of polyphenols on colorectal cancer cells

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Abstract

AIM: To investigate the effects of quercetin and genistein on colon cancer cell proliferation and their estrogen receptor β (ERβ) expression.

METHODS: Colon cancer cells were stably transfected with a mammalian expression vector to overexpress ERβ (HCT8-β8-expressing cells) or a control vector (HCT8-pSV2neo-expressing cells). The proliferation of these cells was examined after treatment with quercetin or genistein (5-100 μmol/L), or 10 nmol/L 17β-estradiol (17β-E2). Cell viability was examined by acridine orange staining following treatments for 48 or 144 h. Effects of quercetin and genistein on ERβ transcriptional transactivation were examined by luciferase activity in HCT8-β8-expressing cells transiently transfected with a pEREtkLUC reporter vector. In addition, the regulation of ERβ transcription by phytoestrogens and 17β-E2 was examined by quantitative polymerase chain reaction.

RESULTS: Proliferation of HCT8-β8-expressing cells was not reduced low doses (5 μmol/L) of quercetin and genistein, while it was reduced at 25-50 μmol/L with an effect similar to 10 nmol/L 17β-E2. Treatment with doses of phytoestrogens ⩾ 75 μmol/L completely blocked cell growth and reduced overall cell counts, however no effects at any dose were observed in HCT8-pSV2neo-expressing cells. These results were supported by viability staining that revealed acridine orange-stained lysosomes with high doses or extended treatment periods. Genistein and quercetin (50 μmol/L) significantly increased ER-responsive luciferase activity similar to 10 nmol/L 17β-E2 ($P < 0.05$). Furthermore, genistein and quercetin (50 μmol/L), as well as 10 nmol/L 17β-E2 significantly increased ERβ mRNA levels in HCT8-β8-expressing cells ($P < 0.05$). In addition, treatment of HCT8-pSV2neo-expressing cells with 50 μmol/L quercetin or 10 nmol/L 17β-E2 significantly increased ERβ mRNA levels compared to untreated controls ($P < 0.05$), though the absolute levels were much lower than in HCT8-β8-expressing cells.

CONCLUSION: The antitumorigenic effects of the phytoestrogenic compounds quercetin and genistein on colon cancers cells occur through ERβ activity and expression.

Key words: Estrogen receptor; HCT8-β8 cells; HCT8-pSV2neo; Quercetin; Genistein

Core tip: Colorectal cancer is one of the most common malignancies worldwide, though its incidence is lower in regions with a high dietary intake of estrogenic polyphenols. Moreover, the expression of estrogen receptor β (ERβ) is high in healthy colonic mucosa, and declines with the progression of colorectal cancer. This study examined the *in vitro* effects of two estrogenic polyphenols, quercetin and genistein, demonstrating their antiproliferative effects and regulation of ERβ activity and expression in colon cancer cells. These data suggest that a possible mechanism for the protective effects of such compounds is through activation and expression of ERβ.

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INTRODUCTION

Colorectal cancer (CRC) is one of the most common malignancies and a leading cause of cancer deaths for both men and women in Western countries[1]. The five-year survival rate remains poor despite significant advances in diagnosis and therapy. CRC results from an interaction among several factors, including lifestyle, family history and diet[2,3]. Since Lacassagne's work in 1955 demonstrating that estrogen administration increases the incidence of mammary cancer in mice[4], many studies have shown the involvement of sex hormones in the risk and development of many types of cancer, including breast cancer and CRC. The incidence of CRC is slightly lower in women compared to men of a similar age[5], and epidemiologic studies and results of the Women's Health Initiative clinical trial show that the risk is reduced in women who take hormone replacement therapy[6]. Furthermore, reduced serum levels of estradiol are associated with downregulated estrogen receptor (ER) expression in the colonic mucosa and a significantly increased risk of CRC[3,7].

ERα and ERβ are the two known subtypes through which estrogens exert their effects on various tissues. Experimental data show differential expression of these receptors, with very low levels of ERα either in normal or pathologic colonic mucosa (adenoma and carcinoma)[8], and high ERβ expression in healthy colonic mucosa, which decreases with the progression of CRC[8-11]. This has led to the proposal that ERβ functions as a tumor suppressor, protecting cells against malignant transformation, and is responsible for the protective effect of estradiol on CRC[12,13].

There is evidence that some polyphenols produced by plants have estrogen-like activity. It has been demonstrated that these phytoestrogens, with molecular structures similar to steroids, could be critical modulators of the human hormonal system and exert hormonal actions on target tissues[14,15]. Phytoestrogens have been widely studied for their potential therapeutic use in the prevention of different diseases and some carcinomas, given that they show some of the protective effects of estrogens in absence of the side effects associated with estrogen administration[16]. These effects may occur through binding to ERs or interacting with enzymes involved in sex steroid metabolism and biosynthesis[17]. Most phenolic compounds show a chemical structure similar to 17β-estradiol (17β-E2), suggesting they might compete for ER binding. However, phytoestrogens can produce estrogenic, anti-estrogenic and unique effects independent from estrogen binding recognition. These diverse actions of phenolic compounds are also tissue-specific, and thus are defined as selective estrogen receptor modulators[18].

Genistein is a phytoestrogen found in soy that may inhibit cancer progression by inducing apoptosis or inhibiting proliferation, the mechanisms by which are a subject of considerable interest[19]. A negative correlation was observed between the incidences of breast, prostate and colon cancer and the phytoestrogen-rich soy diet of some ethnic groups in Asia[20,21]. Recently, several studies have identified a dualistic mode of action by genistein in relation to cancer cell proliferation and cancer risk[22].

Whereas low concentrations of genistein have been shown to enhance the proliferation of breast cancer cells *in vitro*, high concentrations can inhibit their growth[23]. It is possible that the opposing effects of phytoestrogens depend on which ER isoform they interact with.

To better understand the influence of phytoestrogens on cancer development and progression, colon cancer cells were evaluated after exposure to genistein or quercetin, a flavonoid ubiquitously present in many fruits, vegetables, seeds, nuts, olive oil, tea and red wine[24] that also has potentially beneficial effects on cancer prevention[25-27]. The effect of these treatments on ERβ activation and expression, cell growth and cell viability, determined by staining with lysosomotropic acridine orange (AO) to detect lysosomal activation[28-30], were evaluated.

MATERIALS AND METHODS

Cell lines and chemicals

The human colon cancer HCT8 cell line[31,32] was obtained from the American Type Culture Collection (Rockville, MD, United States of America). Cells overexpressing human ERβ (HCT8-β8) were established *via* a stable transfection with the mammalian expression vector pCXN2-hERβ or a control pSV2neo vector (HCT8-pSV2neo)[33]. Genistein, quercetin and 17β-E2 (internal positive control) were purchased from Sigma-Aldrich (St. Louis, MO, United States). Solutions of 17β-E2 and phytoestrogens were dissolved in ethanol and then diluted in cell culture medium to the final concentrations.

Cell culture

Cells were cultured in RPMI 1640 medium (Lonza Group, Basel, Switzerland) supplemented with 10% fetal bovine serum (FBS) or FBS-stripped serum (SFBS; Biological Industries, Kibhutz Beit Haemek, Israel), without phenol red, with 1 mmol/L sodium pyruvate, 2 mmol/L L-glutamine, 100 μg/mL penicillin, 100 μg/mL streptomycin and 280.25 μg/mL Geneticin (G418; Invitrogen of Thermo Fisher Scientific Inc., Waltham, MA, United States) at 37 ℃ with 5% CO_2 humidified air. Confluent cell cultures were detached with a trypsin/ethylenediaminetetraacetic (EDTA) acid solution (Lonza Group) and plated at the desired density in the appropriate medium.

Cell proliferation analysis

For cell proliferation analysis, HCT8-β8- or HCT8-pSV2neo-expressing cells were plated on 6-well plates at a density of 5×10^3 cells/well. After 2 h, the medium was replaced with SFBS medium (phenol red-free medium supplemented with 10% SFBS, and penicillin-streptomycin) and stimulated with genistein or quercetin (5, 25, 50, 75, 100 μmol/L), or with 10 nmol/L 17β-E2 (cells without stimuli were used as a control). Cells were detached with trypsin/EDTA and the number was evaluated by a Bürker hemocytometer every 48 h for 8 d. Measurements for each dose at each time point were collected in triplicate and averaged.

AO staining

Following a 48 or 144 h treatment with quercetin, genistein or 17β-E2, HCT8-β8- or HCT8-pSV2neo-expressing cells were washed three times with phosphate buffered saline (PBS) to remove dead cells and serum proteins (cells without stimuli were used as a control). Cells were incubated in a 0.2% AO solution (in PBS, 2 mL/well) in the dark at room temperature for 10 min and washed three times with PBS. The cells were observed in phase contrast and under fluorescence (BP365/FT395/LP397 filter set) with an Axiovert 200 M microscope and images were acquired with Axiovision Software on an AxioCam HRC 12 megapixel camera (Carl Zeiss, Oberkochen, Germany). When stained with AO, DNA and mitochondria emit green fluorescence (530 nm) and lysosomes emit red fluorescence (650 nm) following excitation by ultraviolet (UV) light (365 nm).

Luciferase assay

HCT8-β8- or HCT8-pSV2neo-expressing were plated on 24-well plates at 2×10^4 cells/well in complete RPMI 1640 culture medium with 10% FBS and penicillin-streptomycin. Twenty-four hours later, the medium was replaced with phenol red-free medium supplemented with 10% SFBS and penicillin-streptomycin. A solution of Attractene Transfection Reagent (Qiagen, Venlo, Limburg, Netherlands) was used to transiently transfect cells with the pEREtkLUC (kindly supplied by Dr. MG Parker)[34] reporter plasmid (395 ng/well) and pERLNULL control plasmid (4 ng/well) (Promega, Madison, WI, United States), and cells were incubated in phenol- and FBS-free RPMI medium for 48 h. After a 24 h stimulation in the same medium with quercetin (50 μmol/L), genistein (50 μmol/L) or 17β-E2 (10 nmol/L) (or no stimulation for controls), whole cell extracts were obtained with the Luciferase Assay System (Promega) and luciferase activity was determined with a luminometer (LKB Instruments, Mount Waverly, Victoria, Australia). Luciferase activity was normalized to β-galactosidase activity measured by a β-gal Assay Kit (Invitrogen) and to total protein concentration. Measurements for each condition were collected in triplicate and averaged.

RNA isolation and real-time quantitative polymerase chain reaction

Total RNA was isolated from cultured cells after stimulation with quercetin (50 μmol/L), genistein (50 μmol/L) or 17β-E2 (10 nmol/L) (from triplicate plates) with TRIzol reagent (Invitrogen) according to the manufacturer's instructions and quantified by UV absorbance. Reverse transcription was performed using the Quantitect Reverse Transcription Kit followed by treatment with ribonuclease-free deoxyribonuclease I (Qiagen). Quantitative polymerase chain reaction (qPCR) was performed using the Kapa Probe Fast qPCR kit (Kapa Biosystems Inc., Wilmington, MA, United States) according to the manufacturer's instructions. Briefly, reactions consisting of 2 μL cDNA, 10 μL KAPA PROBE FAST qPCR Master Mix, 2 μL gene specific primers (10 μmol/L), 1 μL TaqMan Probe (5 μmol/L), and 5 μL RNase-free H_2O were heated at 95 ℃ for 5 min and amplified by 35 cycles of 95 ℃ for 10 s, and 60 ℃ for 30 s using a Rotor-Gene Q (Qiagen). The results obtained were normalized to a housekeeping gene (*RPS18*).

The following primers and corresponding TaqMan probes were used: ERβ: (forward) 5'-TCGCCAGTTATCACATCTGTATGCGG-3', (reverse) 5'-GTGTCTCTCTGTTTACAGGTAAGGTGTG-3', (probe) F/TCCCTGGTG/ZEN/TGAAGCAAGATCGCTAGAA/Q; RSP18: (forward) 5'-CTTCCACAGGAGGCCTAC-3', (reverse) 5'-GATGGCAAAGGCTATTTTCCG-3', (probe) F/TTCAGGGAT/ZEN/CACTAGAGACATGGCTGC/Q.

Statistical analysis

Statistical differences between groups were analyzed in Microsoft Excel (Microsoft, Redmond, WA, United States) using Student's *t*-tests. Data are expressed as mean ± SD. Statistical differences for cell proliferation analysis between treated groups *vs* controls were analyzed in Excel using a parallelism test for linear regression.

RESULTS

Effects of genistein and quercetin on colon cancer cell proliferation

Cell counts of HCT8-β8- or HCT8-pSV2neo-expressing cells cultured with genistein, quercetin or 17β-E2 were performed every 48 h for up to 12 d to assess cell proliferation. Results show that both phytoestrogens dose-dependently significantly reduced the proliferation of HCT8-β8-expressing cells (Figure 1A and B). The inhibition of cell growth by genistein and quercetin was apparent at concentrations of 25 μmol/L, similar to the effects 10 nmol/L 17β-E2. However, higher concentrations of the phytoestrogens (75 and 100 μmol/L) prevented proliferation and reduced overall cell counts. In contrast, quercetin, genistein and 17β-E2 treatments had no effect on the proliferation of HCT8-pSV2neo-expressing cells (Figure 1C and D).

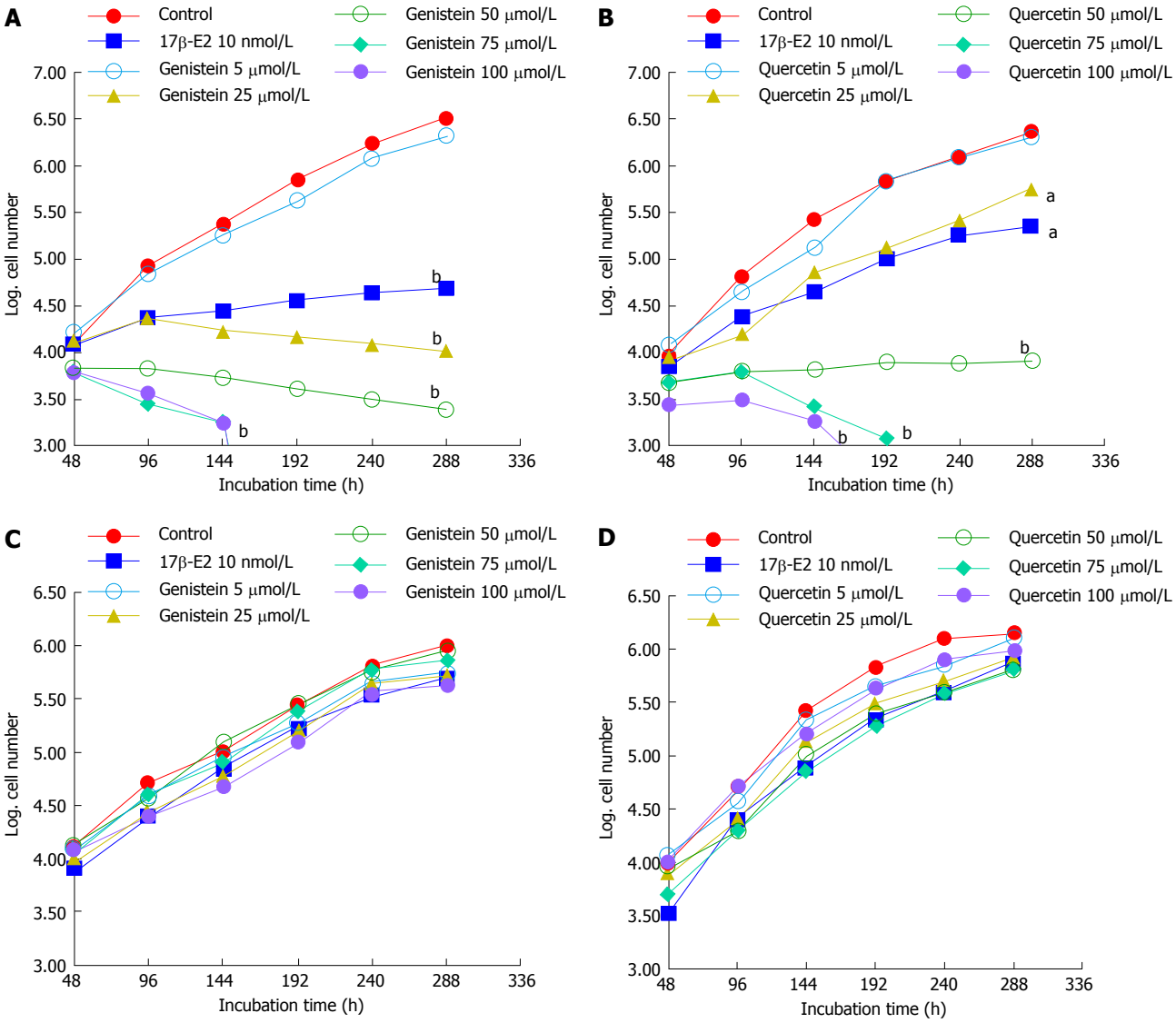

Figure 1 Effects of polyphenols on cell growth. A: Growth of HCT8-â8-expressing cells in the presence of genistein and 17â-E2; B: Growth of HCT8-β8-expressing cells in the presence of quercetin and 17β-E2; C: Growth of HCT8-pSV2neo-expressing cells in the presence of genistein and 17β-E2; D: Growth of HCT8-pSV2neo-expressing cells in the presence of quercetin and 17β-E2. Values are the means of triplicates; [a]$P < 0.05$ *vs* control; [b]$P < 0.01$ *vs* control.

Effects of genistein and quercetin on colon cancer cell viability

AO staining of HCT8-β8-expressing cells treated for 48 h with 5-25 μmol/L genistein (Figure 2B and C), 5-25 μmol/L quercetin (Figure 3B and C) or 10 nmol/L 17β-E2 (Figure 4B) revealed a homogenous green brilliant fluorescence, similar to the untreated control cells. However, red lysosomes became apparent with higher doses of both phytoestrogens (≥ 50 μmol/L) (Figures 2D-F, 3D-F), or extended exposure of concentrations ≥ 25 μmol/L (144 h; Figures 2I-L, 3I-L). There were some red-labeled lysosomes observed with 144-h treatment of 10 nmol/L of 17β-E2 (Figure 4D). Long-term treatment with high doses of phytoestrogens (≥ 75 μmol/L) revealed many cells with pale and homogeneous green fluorescence and many brilliant red-orange lysosomes (Figures 2K, L, and 3K, L), which indicate reduced viability and cellular stress. In contrast, HCT8-pSV2neo-expressing cells were largely unaffected by treatment with genistein (Figure 5), quercetin (Figure 6B), or 17β-E2 (Figure 4E-H), but rather exhibited strong, homogeneous green fluorescence with few lysosomes in all the treated samples after 48 and 144 h.

Effects of genistein and quercetin on ERβ transactivation

To determine if the anti-proliferative effects of genistein and quercetin occurred through activation of ERβ, ER-responsive luciferase activity was measured in HCT8-β8-expressing cells transiently transfected with the pEREtkLUC reporter plasmid. Luciferase activity was significantly increased (165%) following 24 h treatment with 10 nmol/L 17β-E2 ($P < 0.05$) (Figure 7). Similarly, treatment with 50 μmol/L genistein and 50 μmol/L quercetin produced an increase in luciferase activity of 158 and 81%, respectively ($P < 0.05$), compared to an un-

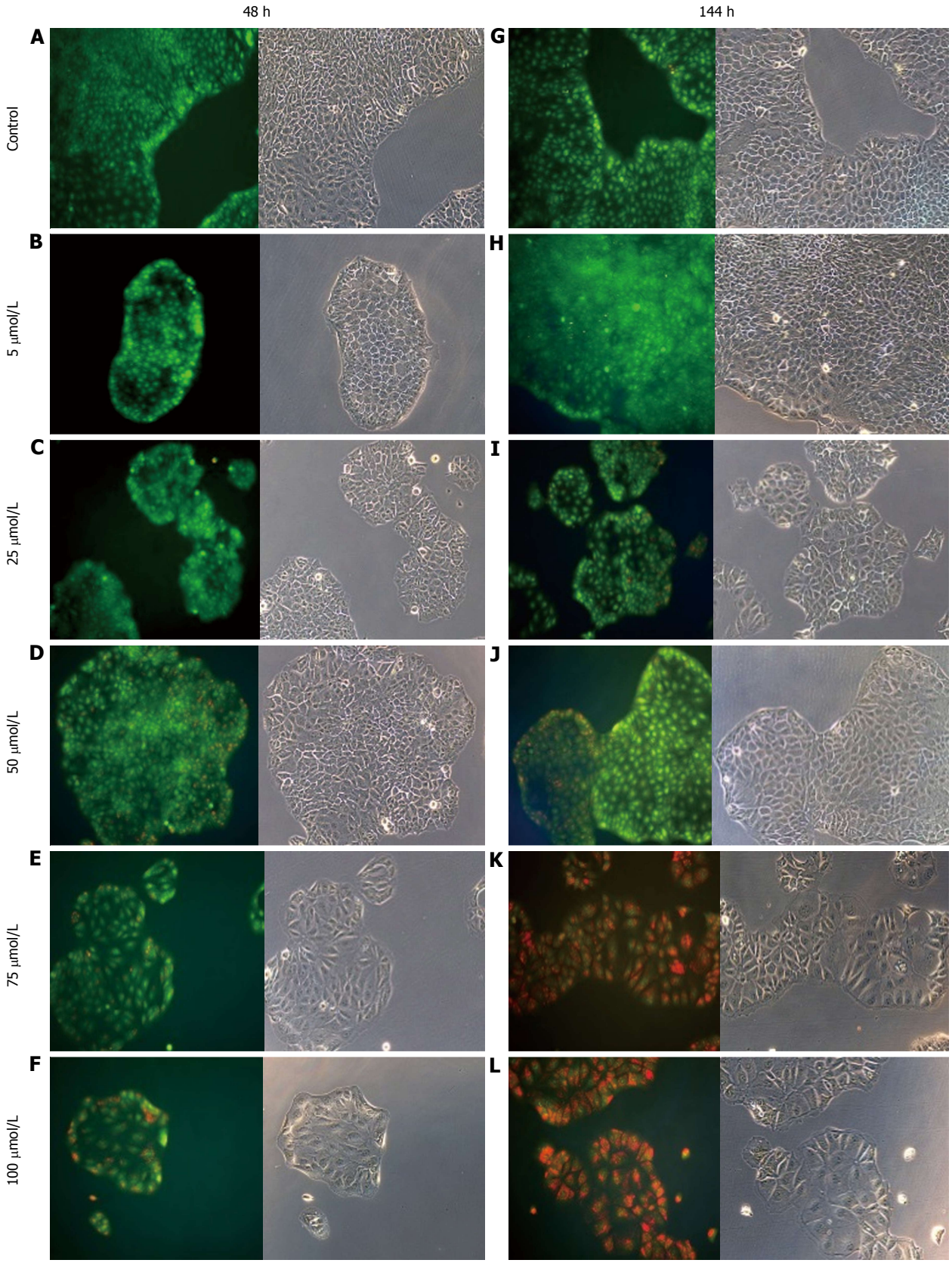

Figure 2 Treatment of HCT8-β8-expressing cells with genistein. HCT8-β8-expressing cells were treated with various concentrations of genistein for 48 h (A-F) or 144 h (G-L) and stained with acridine orange. Nuclei and mitochondria appear green, whereas lysosomes appear red-orange under fluorescence, adjacent to corresponding phase contrast images (magnification × 20).

treated control. ER-responsive luciferase activity was not evaluated for HCT8-pSV2neo-expressing cells as neither of the two polyphenols produced anti-proliferative effects in this cell line.

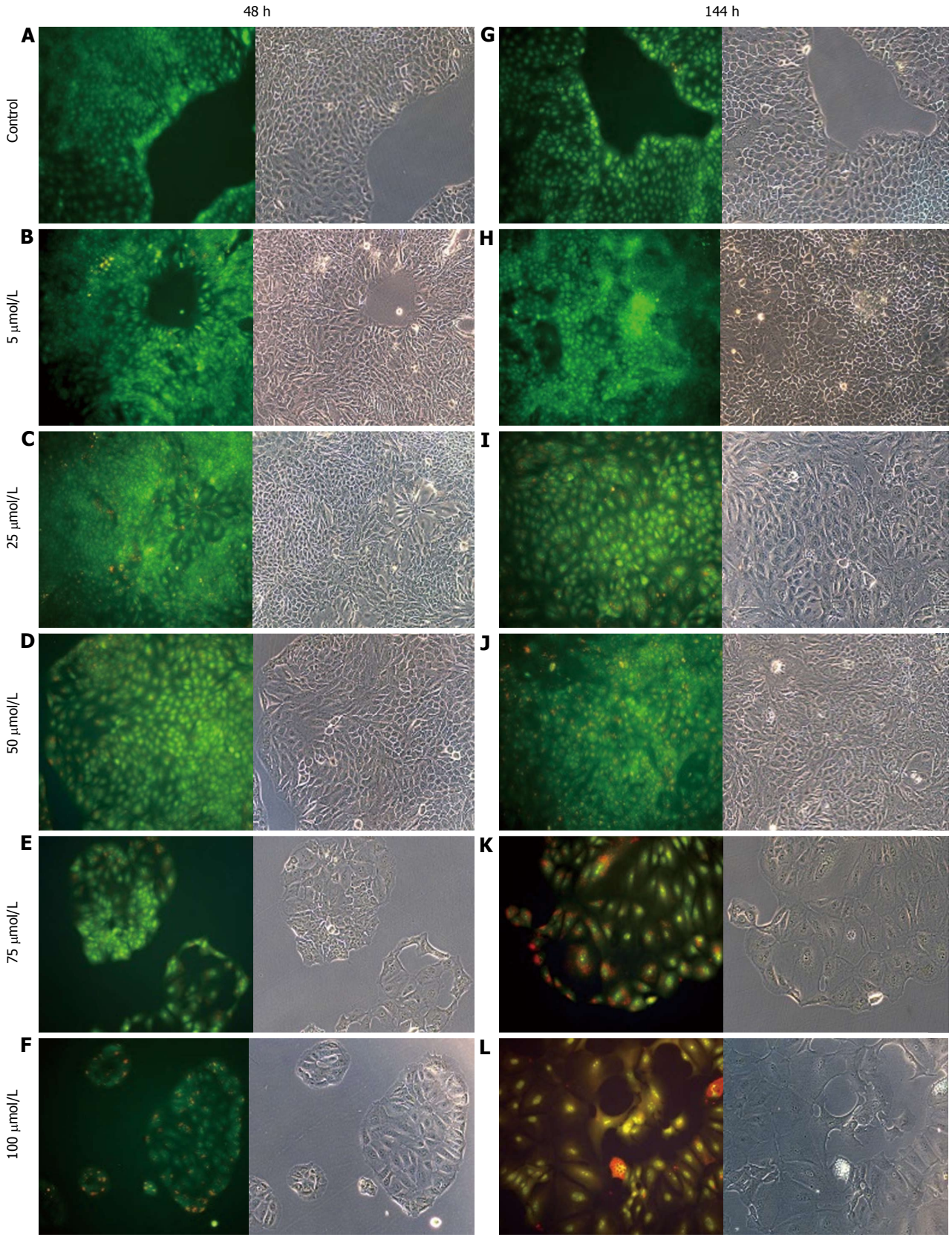

Figure 3 Treatment of HCT8-β8-expressing cells with quercetin. HCT8- β8-expressing cells were treated with various concentrations of quercetin for 48 h (A-F) or 144 h (G-L) and stained with acridine orange. Nuclei and mitochondria appear green, whereas lysosomes appear red-orange under fluorescence, adjacent to corresponding phase contrast images (magnification, × 20).

Effects of genistein and quercetin on ERβ transcription

The expression of ERβ mRNA in HCT8-β8-expressing cells was significantly increased following a six-day treatment with 50 μmol/L genistein ($1.39 \times 10^8 \pm 5.33 \times$

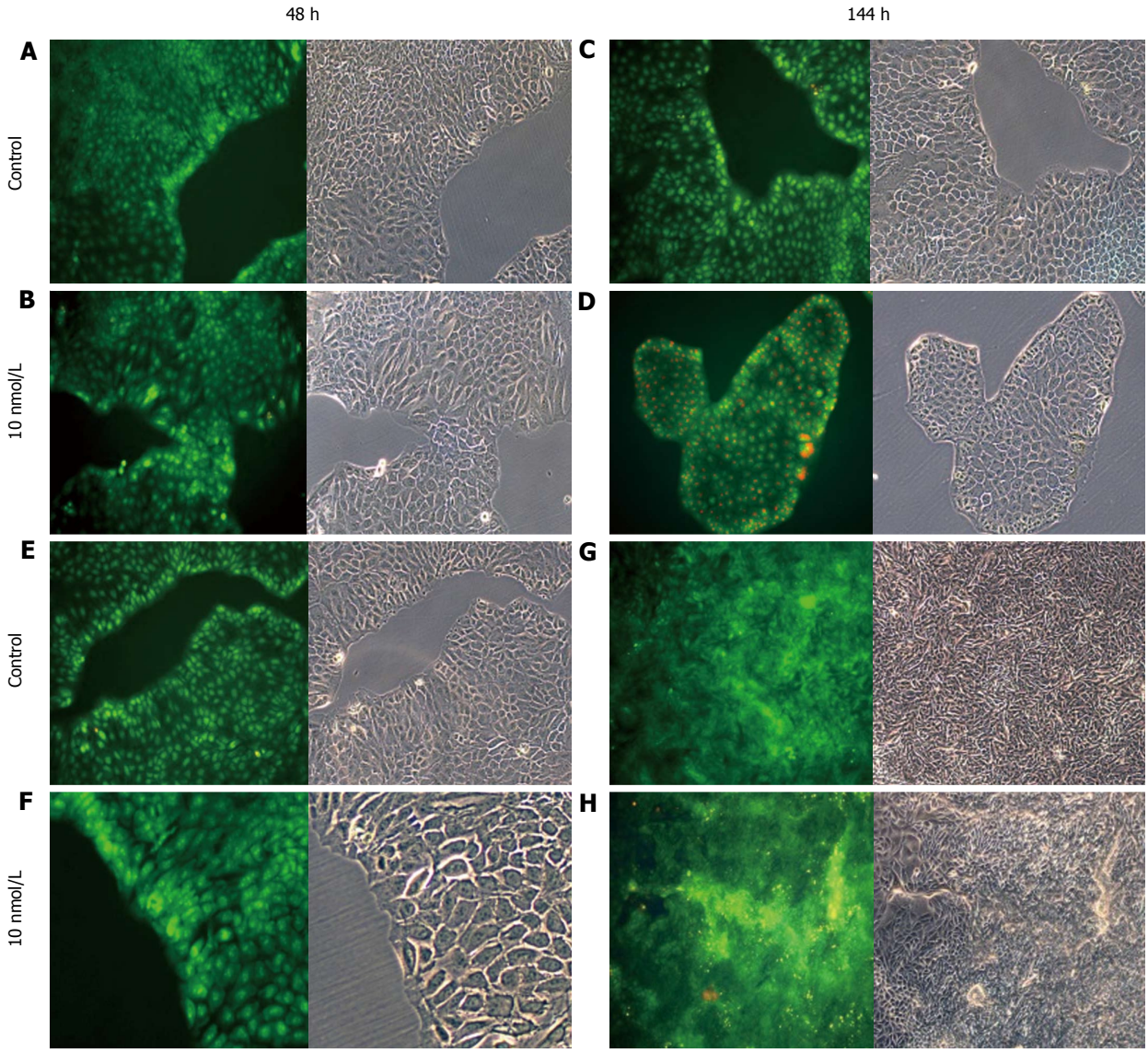

Figure 4 Treatment of cells with 17β-E2. A-D: HCT8-β8-expressing cells; or E-H: HCT8-pSV2neo-expressing cells were treated with 10 nmol/L 17β-E2 for 48 h (A, B, E, F) or 144 h (C, D, G, H) and stained with acridine orange. Nuclei and mitochondria appear green, whereas lysosomes appear red-orange under fluorescence, adjacent to corresponding phase contrast images (magnification × 20).

10^7), 50 μmol/L quercetin ($1.45 \times 10^8 \pm 5.00 \times 10^7$) and 10 nmol/L 17β-E2 ($1.49 \times 10^8 \pm 4.35 \times 10^7$), compared to untreated controls ($5.00 \times 10^7 \pm 1.90 \times 10^7$) (all $P < 0.05$) (Figure 8A). Increases in ERβ mRNA levels were also observed in HCT8-pSV2neo-expressing cells treated with quercetin ($5.88 \times 10^6 \pm 3.20 \times 10^6$) and 17β-E2 ($1.91 \times 10^6 \pm 8.54 \times 10^5$) ($P < 0.05$) (Figure 8B), though the relative expression ($3.97 \times 10^5 \pm 1.37 \times 10^5$) was much lower compared to HCT8-β8-expressing cells.

DISCUSSION

Genistein, found in soybeans and their derivatives, and quercetin, one of the most abundant phytoestrogens in the Western diet[34], are two natural flavonoid molecules with molecular structures similar to 17β-E2, which is a substrate of ERβ. Consumption of phytoestrogen-rich foods is correlated with a reduced incidence of CRC[35,36]. Moreover, plasma concentrations of phytoestrogens are high in populations from China, Japan and countries of Southeast Asia, which are considered to have low risks for malignancy, particularly for hormone-sensitive cancers such as breast cancer, prostate cancer and CRC[20,37,38].

The possible antitumorogenic effects of phytoestrogens were tested in two CRC cell models, including a hormone-sensitive cell line of colon adenocarcinoma expressing very low levels of ERβ (HCT8-pSV2neo-expressing), and the same cell line with high levels of ERβ (HCT8-β8-expressing). The range of phytoestrogen concentrations used were based on epidemiologic and absorption human studies. Quercetin intake is reported to be approximately 16 mg/d[34], and a study by Hollman *et al*[39] found that 76% of orally administered quercetin aglycone is recovered in the ileostomy bags of subjects who underwent a colectomy, which can be considered a model compartment for the colon[40]. Therefore, an aver-

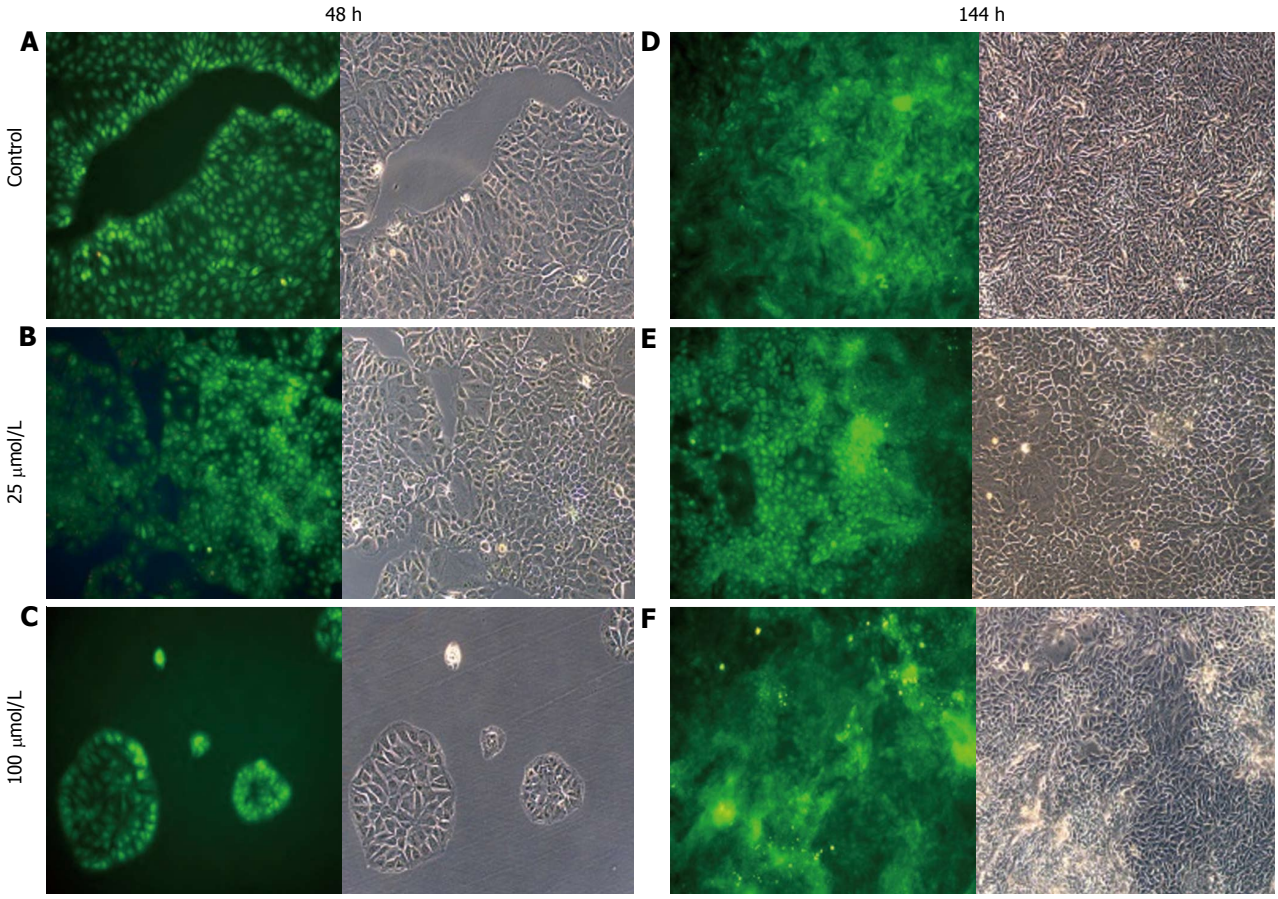

Figure 5 Treatment of HCT8-pSV2neo-expressing cells with genistein. A-F: HCT8-pSV2neo-expressing cells were treated with 25 μmol/L (B and E) or 100 μmol/L (C and F) genistein for 48 h (A-C) or 144 h (D-F) and stained with acridine orange. Nuclei and mitochondria appear green, whereas lysosomes appear red-orange under fluorescence, adjacent to corresponding phase contrast images (magnification × 20).

age 12 mg of quercetin reaches the colon daily, indicating that, depending on dietary intake, quercetin concentrations of 40-80 μmol/L in the colon are likely.

Dietary intakes of 39 and 47 mg of genistein/day for the adult Chinese and Japanese populations, respectively, have been reported[41-43], whereas the Western diet provides only 1-2 mg/d, with values of up to 3-12 mg of genistein/day for those following a vegetarian diet[44,45].

The results of the *in vitro* proliferation analyses show that even relatively low doses of phytoestrogens can reduce, and concentrations comparable to those found in Eastern diets can block, proliferation of HCT8-β8-expressing, but not HCT8-pSV2neo-expressing cancer cells. These data confirm results described in the literature regarding the behavior of the same phytoestrogens on different CRC cell lines, as well as in other hormone-sensitive cancer cells[34,46-48]. For example, genistein has an anti-proliferative effect on the estrogen-dependent human breast cancer MCF-7 cell line similar to that induced by 17β-E2[23], and the proliferation of prostate cancer cells is reduced by quercetin[24]. However, a study on the Caco-2 colon cancer cell line, which contains low levels of ERβ, showed that cell cycle gene expression and cell proliferation was reduced with 50 μmol/L of quercetin, resulting in cell cycle arrest[25,26].

The observed anti-proliferative effects of phytoestrogens on HCT8-β8-expressing cells were accompanied by activation of ERβ, as observed by luciferase activation. The results show that both genistein and quercetin increased luciferase activity, comparable to levels induced by 17β-E2. This activity likely depends directly on ERβ binding, which can then modulatate the expression of specific proteins directly involved in cell cycle regulation[49-55]. Furthermore, the concentrations of quercetin and genistein that inhibited cell growth but did not induce cell death were also found to increase ERβ mRNA levels. The basal level of ERβ in HCT8-β8-expressing cells perpetuated a large increase in mRNA after treatment with both phytoestrogens and 17β-E2. A proportionately larger increase was observed in HCT8-pSV2-neo-expressing cells, though the relative levels were much lower.

Taken together, these data suggest that the inhibition of cell growth, activation of ERβ and the increased transcription of ERβ depend on the binding of phytoestrogens to ERβ, as these effects were absent or minimal in HCT8-pSV2neo-expressing cells, though future experiments with agents blocking the estrogen receptor will be necessary to confirm this. The data presented here are in agreement with observations from other hormone-

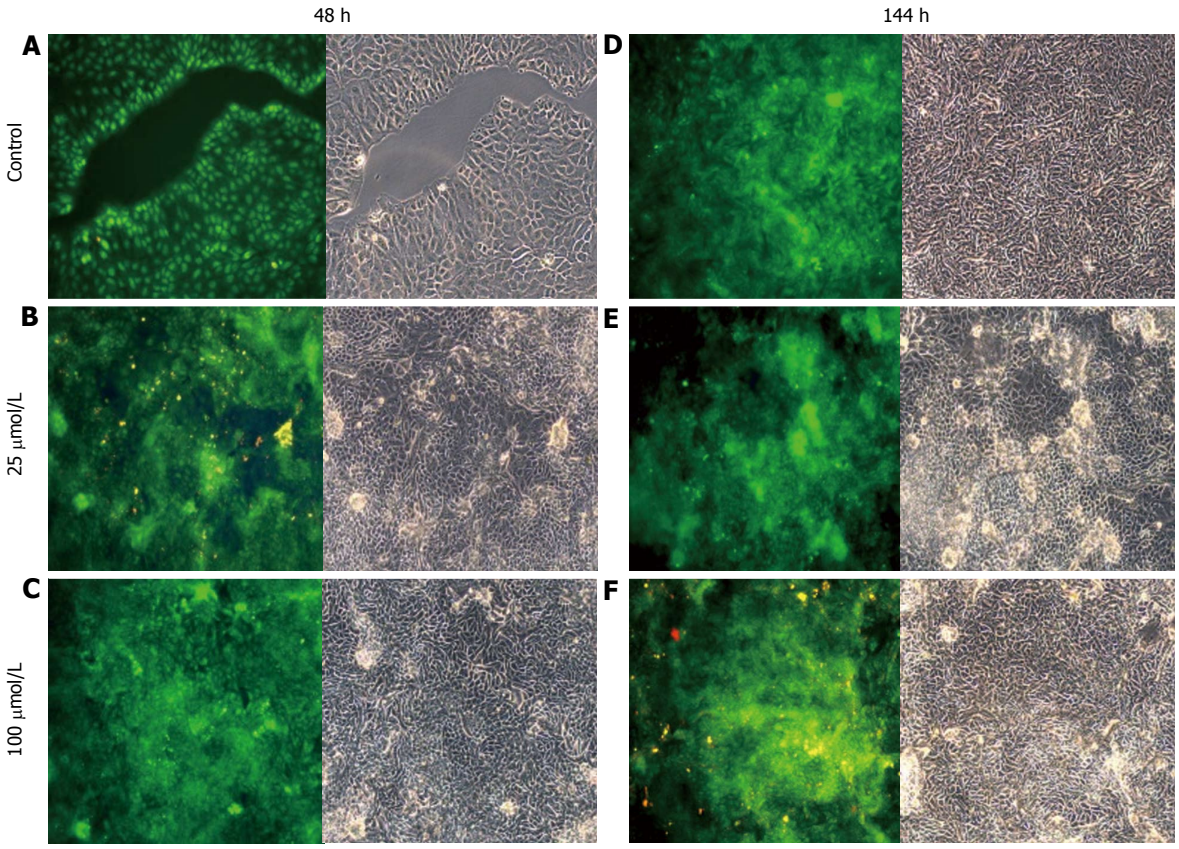

Figure 6 Treatment of HCT8-pSV2neo-expressing cells with quercetin. A-F: HCT8-pSV2neo-expressing cells were treated with 25 μmol/L (B and E) or 100 μmol/L (C and F) quercetin for 48 h (A-C) or 144 h (D-F) and stained with acridine orange. Nuclei and mitochondria appear green, whereas lysosomes appear red-orange under fluorescence, adjacent to corresponding phase contrast images (magnification × 20).

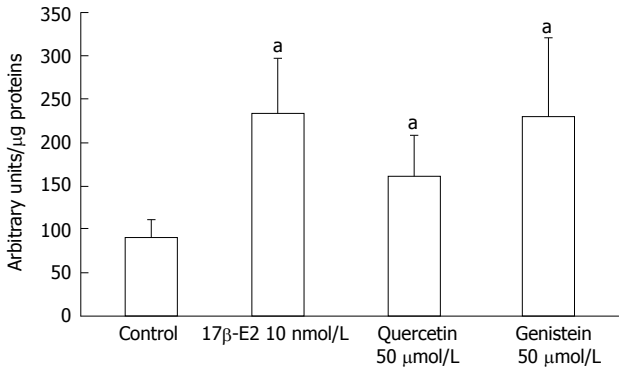

Figure 7 Induction of EREtkLUC reporter gene activity. Treatment of HCT8-â8-expressing cells with 17â-E2, genistein and quercetin induces EREtk expression observed as relative luciferase activity. Values are the mean ± SD of triplicates; $^{a}P < 0.05$ *vs* control.

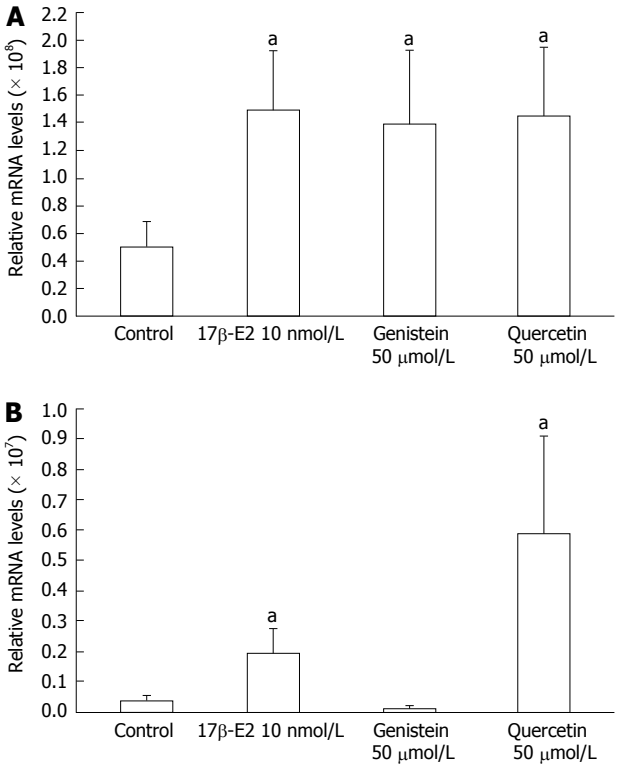

Figure 8 Expression of ERβ mRNA levels by quantitative real-time reverse transcription-polymerase chain reaction. Induction of ERβ expression by 17β-E2, genistein and quercetin in A: HCT8-β8-expressing cells; B: HCT8-pSV2neo-expressing cells. The results are expressed relative to RPS18 mRNA levels. Values are the mean ± SD of quadruplicates; $^{a}P < 0.05$ *vs* control.

sensitive cancers[25,56], and also demonstrate the protective role of ERβ that has been reported for estrogen-sensitive tissue such as breast, ovary, prostate and colorectal mucosa[57-61]. Furthermore, these results support the epidemiologic and experimental data which show the protective action of both the tested phytoestrogens at a concentration similar to the levels in colorectal mucosae that result from daily phytoestrogen intake in the Eastern diet, and indicate that dietary intake of phytoestrogens may protect against CRC by acting on tumoral cell growth and modulating gene transcription. In conclusion, our study indicates that the mechanism for antitumorogenic activity

of phytoestrogens on CRC could involve regulation of ERβ expression.

COMMENTS

Background

Recent evidence suggests a close relationship between estrogen and colorectal cancer (CRC), one of the most common malignancies, such that reduction in circulating levels of estradiol increases the risk of developing cancer. Furthermore, regions with a high dietary intake of phytoestrogens, natural molecules with estrogen-like effects, have lower incidences of CRC. The expression of estrogen receptor β (ERβ), is high in healthy colorectal mucosa, and reduced in cancerous tissue. However, the mechanism regulating the effect of estrogen on the development of CRC is not well understood.

Research frontiers

Among the phytoestrogens examined for their antitumoral functions, the flavonoids genistein and quercetin are the most well studied. In this *in vitro* study, the authors evaluate these two phytoestrogens, which are common in food sources, and suggest that their anti-proliferative effects are through the activation and expression of ERβ.

Innovations and breakthroughs

Several *in vivo* studies have highlighted the protective antitumoral role of two phytoestrogens, quercetin and genistein, in different hormone-sensitive cancers and the protective role of ERβ on estrogen-sensitive tissues such as breast, ovary, prostate and colorectal mucosa. This *in vitro* study confirms epidemiologic and experimental data which show the protective action of these phytoestrogens against CRC, and demonstrate their effect on cancer cell growth and ERβ transcription. In particular, this study reveals that these effects occur at concentrations of quercetin that are equivalent to those obtained following a daily intake of 16 mg/d.

Applications

By studying the influence of phytoestrogens on the growth of colon cancer cells and their regulation of ERβ expression, this study suggests that similar results could also be found for other hormone-sensitive tissues. Furthermore, the results further suggest that an increase in the dietary consumption of foods rich in phytoestrogens could represent a future strategy for the prevention of CRC and other hormone-sensitive cancers.

Terminology

Estrogen receptors ERα and ERβ are activated by 17-estradiol. Phytoestrogens are a group of plant-derived compounds, including flavonoids, coumestans, lignans and stilbenes, with estrogenic properties. Genistein and quercetin are the most representative of the phytoestrogens that have been studied for their antitumorigenic properties.

Peer review

This study examines the biologic effects of two phytoestrogens on cell growth and expression of ERβ in colon cancer cell lines. The results indicate that quercetin and genistein exert their effects by activating and regulating the expression of ERβ. This study has significance for guiding future preventive therapies for colorectal cancer.

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CASE REPORT

Neuroendocrine tumors of the gastrointestinal tract: Case reports and literature review

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Abstract

Neuroendocrine tumors (NET) previously called carcinoid tumors are neoplasms of enterochromaffin/neuroendocrine cell origin which display neurosecretory capacity that may result in the carcinoid syndrome. The annual incidence of patients with NET is 8.4 per 100000; yet many NET remain asymptomatic and clinically undetected. A majority of NET follows a benign course; however, some will display malignant characteristics. NET most commonly occur in the gastrointestinal tract (67%) and bronchopulmonary system (25%). Gastrointestinal NET occur within the stomach, small intestine, liver, and rectum. We report a retrospective study of 11 subjects: Eight with benign carcinoid tumors: duodenal bulb (*n* = 2), terminal ileum (*n* = 1), sigmoid colon (*n* = 2), and rectum (*n* = 3); three with malignant carcinoid: liver (*n* = 1) and intra-abdominal site (*n* = 2). The diagnosis, endoscopic images, outcome, treatment and review of the literature are presented.

Key words: Neuroendocrine; Carcinoid; Gastrointestinal; Tumors

Core tip: Endoscopic procedures sometimes reveal submucosal lesions within the gastrointestinal tract that are resected and confirmed as neuroendocrine tumors by appropriate immunochemical stains. Most will be benign as demonstrated in our series of 11 subjects. This case series of gastrointestinal neuroendocrine tumors reminds every endoscopist to carefully examine the upper and lower gastrointestinal tract for such lesions.

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INTRODUCTION

Historically described as a more indolent behaving tumor than adenocarcinoma by Oberndorfer in Germany in 1907, neuroendocrine (carcinoid) tumors (NET) are undergoing a location change within the gastrointestinal tract[1-4]. A shift in the anatomic location has occurred over the last half-century. Data from 1950 to 1971 identified the appendix as the most common site followed by rectal and ileum for NET[4]. However, a recent evaluation of carcinoid tumors identified in the Surveillance, Epidemiology and End Results Program between 1973

Table 1 Clinical data of patients with neuroendocrine tumors

Patient (age, yr/sex)	Initial evaluation	Site	Diagnostic studies	Outcome
65/F	Hematochezia, IBD epigastric pain	Duodenal bulb	12 21 05 EGD duodenal bulb polyp; path: neuroendocrine tumor 12 30 05 repeat EGD, no residual, path: neuroendocrine tumor 11 24 08 repeat EGD no recurrence, COL mucosal prolapse syndrome	Alive and well
59/M	GERD with break-through symptoms	Duodenal bulb	11 11 08 EGD duodenal bulb polyp, path: neuroendocrine tumor 12 22 08 EGD, no residual tumor 12 30 08 PET scan negative	Alive and well
50/F	2nd opinion for liver metastatic disease	Liver	02 09 04 EGD chronic esophagitis, HH, fundic nodularity, path: benign lymphoid aggregates 03 16 04 PET/CT innumberable larg hepatic lesions replacing R and L lobes consistent with neuroendocrine tumor	Expired 12 04
70/M	Epigastric pain and 15 lb weight loss	Intra-abdominal	04 15 08 EGD chronic esophagitis, HH, acute and chronic gastritis; path: reactive gastropathy; COL: 1 adenomatous/2 hyperplastic polyps 04 16 08 CT Abd/Pelvis mesen-teric mass 04 24 08 CT guided bx: path: neuroendocrine tumor	05 08 treated with sandostatin
46/F	Nausea, vomiting, abdominal pain	Intra-abdominal	01 02 10 CT Abd/Pelvis ascites small bowel and colonic obstruction 01 04 10 Gastrografin emema sigmoid Obstruction 01 04 10 exploratory laparotomy desmoplastic reaction, sigmoid colon with liver metastases and intraperitoneal implants; bx of implants positive for chromogranin and synapotophysin 01 19 10 COL 3 cm stenosis at 30 cm due to extrinsic pressure; stent placed 01 26 10 serum CGA, 27 nmole/L	Discharge To hospice
40/M	Recurrent perianal abscess r/o IBD	Terminal ileum	12 05 06 COL 10 mm sessile polyp in terminal ileum, path: neuroendocrine tumor	Lost to follow-up
50/F	GERD and CRCS	Sigmoid	04 04 08 EGD chronic esophagitis, HH, path: mild reactive gastropathy, COL 4 mm sigmoid neuroendocrine tumor resected 04 30 08 normal octreotide scan 03 30 09 COL negative bx at prior polypectomy site	Alive and well
75/F	Breast cancer and CRCS	Sigmoid	02 06 08 COL 7 mm sigmoid submucosal nodule resected; cells positive for synaptophysin, but negative for chromogranin 03 11 08 Urinary 5-HIAA negative 04 22 08 Repeat COL with resection of remaining neuroendocrine tumor 05 19 09 COL negative for recurrence	Alive and well
55/M	LLQ tenderness, CRCS	Rectum	08 22 06 COL sigmoid tubulovillous adenoma and 6 mm rectal neuro-endocrine tumor 09 01 09 COL hyperplastic polyp, no recurrence of neuroendocrine tumor	Alive and well
55/F	CRCS	Rectum	05 01 09 COL 8 mm neuroendocrine tumor COL 1 yr later no recurrence	Alive and well
60/F	CRCS	Rectum	11 29 07 COL submucosal nodule neuroendocrine tumor 01 28 08 COL no recurrence	Alive and well

IBD: Inflammatory bowel disease; CGA: Chromogranin A; EGD: Esophagoduodenoscopy; COL: Colonoscopy; GERD: Gastroesophageal reflux disease; HH: Hiatal hernia; R: Right; L: Left; bx: Biopsy; CRCS: Colorectal cancer screening; F: Female; M: Male; 5-HIAA: 5-hydroxyindoleacetic acid; PET/CT: Positron emission tomography/computed tomography; LLQ: Left lower quadrant.

and 1999 found the ileum to be the most frequent site of gastrointestinal NET followed by the rectum; the appendix accounted for only 4.8% of NET[4]. Additionally, gastric NET accounted for an increasing proportion of gastrointestinal NET[4,5]. This change in location of NET has resulted from changes in diagnostic modalities used as well as reporting techniques over time[6]. The estimated incidence in the United States ranges from 2.5-5 cases per 100000[4]. A European investigation which included both surgical and autopsy specimens, reported an overall incidence of 8.4 cases per 100000[4,7,8]. Incidence estimates are limited by the clinically silent nature of many NET which remain undetected until autopsy[6].

CASE REPORT

This case series describes a wide spectrum of benign gastrointestinal NET originating in the small intestine (n = 2), terminal ileum (n = 1), colon (n = 2), rectum (n = 3), malignant NET of the liver (n = 1) and intraabdominal sites (n = 2) (Table 1).

Patient 1

A 65-year-old female with a history of possible inflam-

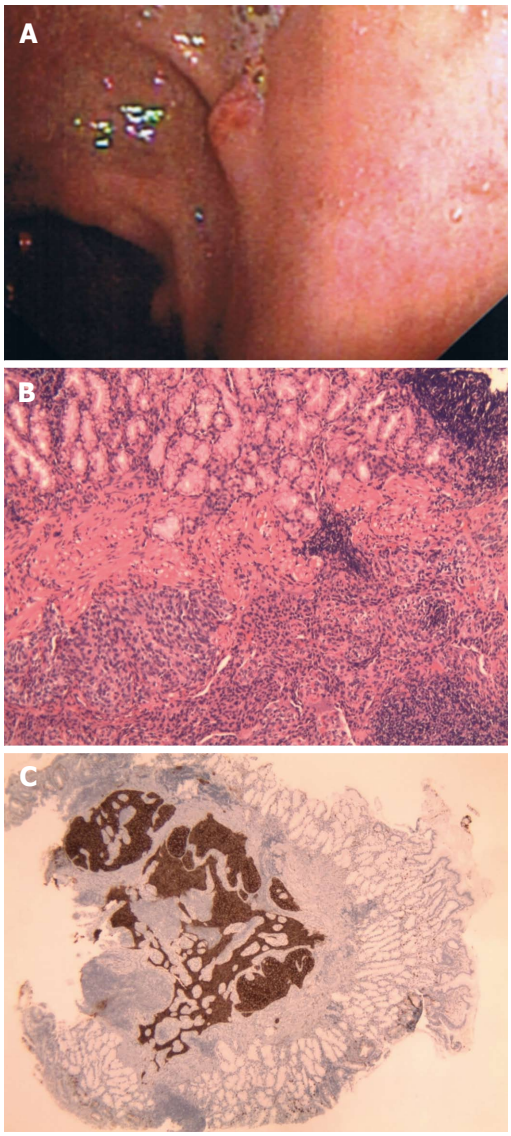

Figure 1 A 65-year-old female with a history of possible inflammatory bowel disease presented for evaluation of epigastric pain and occasional hematochezia. A: Patient 1, neuroendocrine (carcinoid) tumors as duodenal nodule at endoscopy; B: Solid growth pattern with organoid architecture and bland monotonous cells with lack of significant atypia and increased mitoses. H and E, × 10; C: Neoplastic neuroendocrine cells show diffuse positivity for Chromogranin. Chromogranin, × 20.

matory bowel disease presented for evaluation of epigastric pain and occasional hematochezia. Colonoscopy revealed multiple polypoid lesions throughout the colon with biopsies consistent with mucosal prolapse syndrome. Esophagogastrastroduodenoscopy (EGD) revealed mild esophagitis, chronic gastritis, and a 5 mm polyp in the duodenal bulb biopsied with cold forceps (Figure 1A). Pathology demonstrated duodenal mucosa with atypical organized nests of cells with expression of low molecular cytokeratin, neuron-specific enolase (NSE), chromogranin, and synaptophysin on immunohistochemistry consistent with a neuroendocrine tumor (Figure 1B and C). Repeat EGD was performed 35 mo later and revealed no residual neuroendocrine tumor.

Patient 2

A 61-year-old male with a history of gastroesophageal reflux disease (GERD) underwent EGD for evaluation of chest discomfort with breakthrough reflux symptoms while taking a proton pump inhibitor daily. LA Grade C esophagitis and ulcerated mucosa were present in the distal esophagus. A 6 mm sessile polyp also observed in the duodenal bulb and resected by snare. Pathology revealed a neuroendocrine tumor of the duodenum. Positron emission tomography-computed tomography (PET-CT) was performed and demonstrated no evidence of hypermetabolic malignancy. A repeat EGD with biopsies from the previous polypectomy site six weeks later demonstrated reactive duodenopathy with foveolar metaplasia but no residual neuroendocrine tumor.

Patient 3

A 50-year-old female with chronic diarrhea was found to have metastatic liver disease of unknown primary origin on CT. The largest lesion measured 9 cm × 6 cm in the right hepatic lobe and PET-CT demonstrated only moderate metabolic activity consistent with a neuroendocrine tumor. CT-guided liver biopsy demonstrated metastatic neuroendocrine tumor with positive synaptophysin, chromogranin, NSE, and CD57 reactions on immunohistochemistry. EGD was performed that showed chronic esophagitis, hiatal hernia, and nodularity in the gastric fundus. Pathology from gastric biopsies revealed only benign lymphoid aggregates. Follow-up CT findings included a 2.4 cm partially calcified mass in the mid-abdominal mesentery suggestive of a neuroendocrine tumor of small bowel origin. The patient was started on long-acting octreotide and entered into hospice care 28 mo after initial presentation.

Patient 4

A 70-year-old male presenting with epigastric pain and 15 pound weight loss underwent upper endoscopy revealing chronic esophagitis, hiatal hernia, acute and chronic gastritis involving the antrum, and a small polypoid lesion which was found in the duodenal bulb. Biopsies were consistent with chronic duodenitis. Colonoscopy revealed one tubular adenoma < 1 cm and multiple hyperplastic polyps. A 3 cm mesenteric mass with surrounding desmoplastic reaction, small bowel thickening, and a 2 cm liver lesion were found on CT of the abdomen and pelvis (Figure 2A). CT guided biopsy of the mesenteric mass demonstrated a metastatic well-differentiated neuroendocrine tumor with immunohistochemistry positive for cytokeratin, NSE, synaptophysin, chromogranin, and CD56 (Figure 2B and C); however, biopsy of the liver lesion was negative for malignancy. PET-CT demonstrated heterogenous metabolic activity of the mesenteric mass with metabolic activity of the liver lesion similar to the surrounding hepatic parenchyma. Urinary 5-hydroxyindoleacetic acid (5-HIAA) was within normal range. The overall presentation was most consistent with a neuroen-

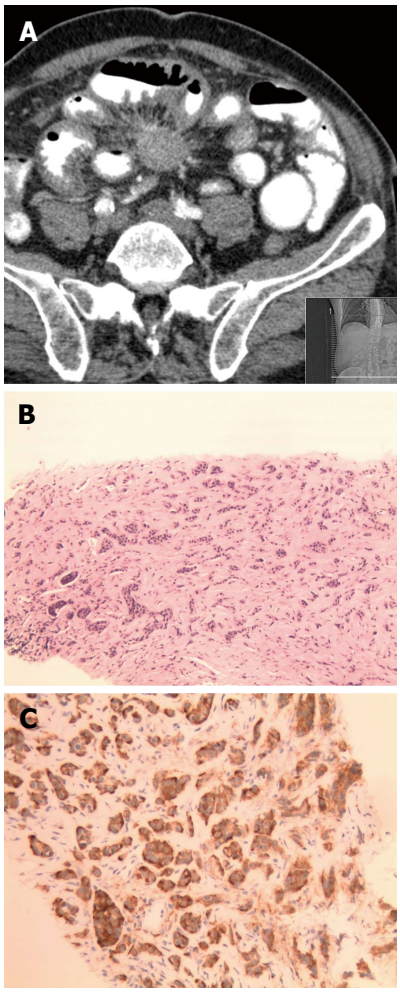

Figure 2 A 70-year-old male presenting with epigastric pain and 15 pound weight loss underwent upper endoscopy revealing chronic esophagitis, hiatal hernia, acute and chronic gastritis involving the antrum, and a small polypoid lesion which was found in the duodenal bulb. A: Patient 4, neuroendocrine (carcinoid) tumors as solid spiculated mesenteric mass on computed tomography of abdomen; B: Diffuse infiltration by monotonous bland cells with trabecular growth pattern. Mitoses, atypia and necrosis are not identified. H and E, × 10; C: The tumor cells are diffusely and strongly positive for CD56 immunohistochemical stain. CD56, × 20.

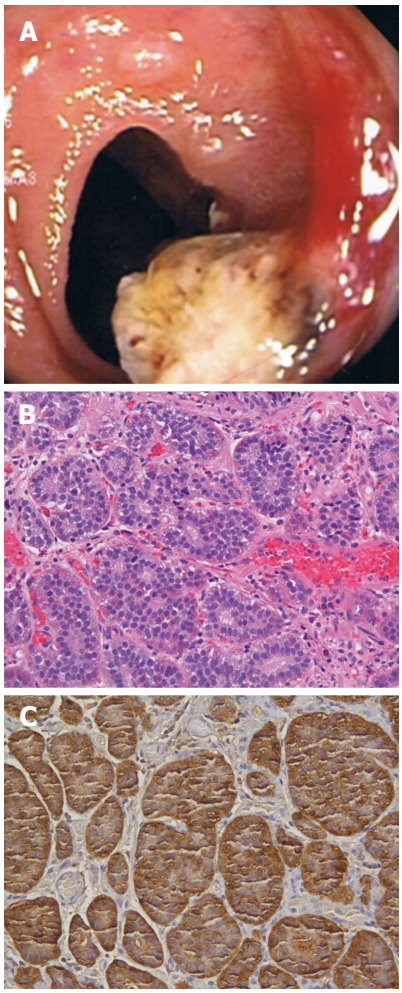

Figure 3 A 40-year-old male with recurrent perianal fistulous disease underwent colonoscopy to rule out inflammatory bowel disease. A: Patient 6, neuroendocrine (carcinoid) tumors as 10 mm ileocecal sessile polyp at colonoscopy; B: Nests of monotonous cells with bland nuclei arranged in organoid pattern. H and E, × 10; C: Carcinoid tumor; Chromogranin A: Marked cytoplasmic positivity.

docrine tumor originating in the small bowel. The patient was started on long-acting octreotide therapy and did not undergo surgical resection of the tumor.

Patient 5

A 45-year-old female presented to an outside facility with nausea, vomiting, and abdominal pain and had dilation of the small bowel and colon and ascitic fluid on CT scan. Gastrografin enema demonstrated an obstruction in the sigmoid colon. An area of desmoplastic reaction involving the sigmoid colon was found during exploratory laparotomy along with multiple metastatic lesions to the liver and mesenteric and peritoneal implants. Surgical decompression of the small bowel and colon was performed and the patient was transferred for further care. Biopsies obtained from the peritoneal implants were consistent with a low-grade neuroendocrine tumor with immunohistochemistry positive for chromogranin and synaptophysin. Serum chromogranin A level was elevated at 27 nmol/L. Following transfer to our facility, the patient underwent colonoscopy which revealed a 3 cm area of stenosis due to extrinsic compression 30 cm from the anal verge. As no further surgical intervention was deemed appropriate, two overlapping metal colonic stents (Wallstent, 22 mm × 90 mm and 22 mm × 60 mm) were placed across the area of stenosis. The patient was later discharged for hospice care.

Patient 6

A 40-year-old male with recurrent perianal fistulous disease underwent colonoscopy to rule out inflammatory bowel disease. Colonoscopy revealed normal colonic mucosa and a 1 cm sessile polyp at the terminal ileum (Figure 3A). Snare polypectomy was performed and pathology revealed a submucosal neuroendocrine tumor with well formed nests of cells and diffuse expression of synaptophysin and chromogranin. KI-67 proliferative index was < 5% (Figure 3B and C). The patient was lost to follow-up.

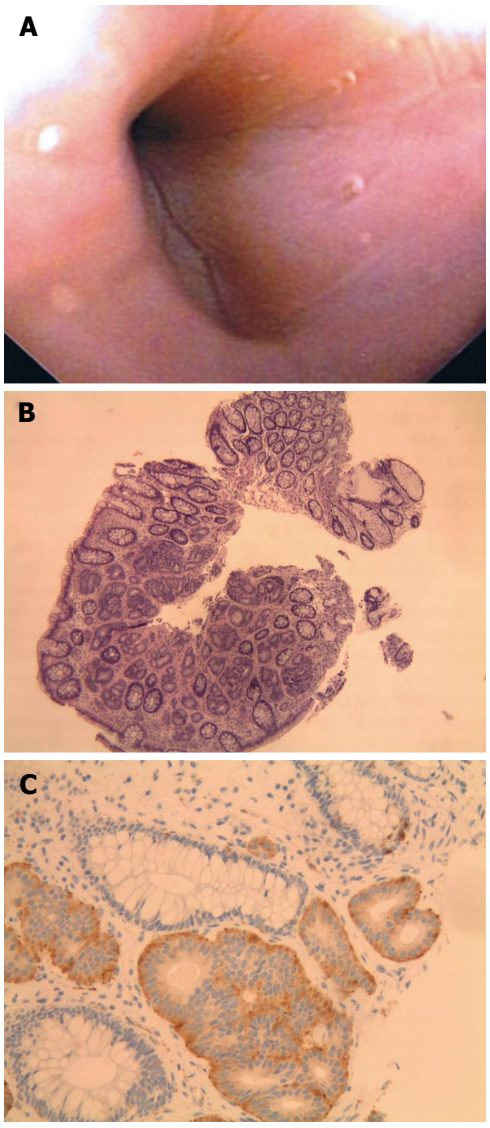

Figure 4 A 50-year-old female presented for evaluation of gastroesophageal reflux disease and colon cancer screening. A: Patient 7, neuroendocrine (carcinoid) tumors as sessile sigmoid polyp at colonoscopy; B: Organoid growth pattern with regular bland nuclei with indistinct cell borders. H and E, × 10; C: The neuroendocrine cells are positive for Synaptophysin and adjacent colonic glands are negative. Synaptophysin, × 20.

Patient 7

A 50-year-old female presented for evaluation of GERD and colon cancer screening. EGD revealed a hiatal hernia, chronic esophagitis, and chronic gastritis. On colonoscopy, benign polyps were removed from the cecum and transverse colon. A 5 mm sessile polyp resected with hot forceps in the sigmoid colon (Figure 4A); pathology demonstrated atypical proliferation of cells and glandular-like inflammation with monotonous nuclei indicative of a neuroendocrine tumor. Immunostains were positive for chromogranin, synaptophysin, and CD56 consistent with a neuroendocrine tumor (Figure 4B and C). Somatostatin receptor scintigraphy demonstrated no evidence of other carcinoid tumors. Surveillance colonoscopy performed one year later revealed a scar at the site of the previously resected tumor without tumor recurrence.

Patient 8

A 77-year-old female with Stage I right breast cancer presented for screening colonoscopy. A 7 mm submucosal nodule was biopsied from the sigmoid colon; pathology revealed tumor cells positive for synaptophysin and negative for chromogranin but overall consistent with a neuroendocrine tumor. Urinary 5-HIAA levels and octreotide scan were unremarkable. Endoscopic mucosal resection was subsequently performed with a snare. Excisional biopsy consisted of a 7 mm × 6 × mm × 3 mm submucosal neuroendocrine tumor. Colonoscopy one year later revealed no recurrence.

Patient 9

A 55-year-old male presented for evaluation of left lower quadrant tenderness and colon cancer screening. Colonoscopy revealed a 1.4 cm tubulovillous adenoma in the sigmoid colon. A 6 mm rectal polyp removed by snare was consistent with a neuroendocrine tumor. Surveillance colonoscopy three years later revealed a 6 mm hyperplastic polyp in the rectum and no evidence of recurrence of a neuroendocrine tumor.

Patient 10

A 54-year-old female presented for colon cancer screening. On colonoscopy, an 8 mm nodule was found in the rectum. Snare polypectomy was performed. Pathology demonstrated atypical proliferation of cells and glandular-like inflammation with monotonous nuclei suggestive of a neuroendocrine tumor. Colonoscopy one year later was negative for recurrence.

Patient 11

A 60-year-old female presented for colon cancer screening. On colonoscopy, a 5 mm submucosal nodule was found in the rectum and removed snare polypectomy. The biopsy was consistent with a neuroendocrine tumor involving the submucosa with tumor cells positive for synaptophysin and focally positive for chromogranin. Fourteen months later, colonoscopic biopsies from the polypectomy site revealed no recurrence.

DISCUSSION

Our case series describes a wide spectrum of benign gastrointestinal NET originating in the small intestine, colon, and rectum and malignant NET originating in the liver and intraabdominal sites. The following discussion will focus on the diagnosis and management of NET originating from the luminal gastrointestinal tract and will not include pancreatic NET.

Advances in our understanding of both the biologic and morphologic heterogeneity of NET have left the term "carcinoid" nearly obsolete[7]. Gastroenteropancreatic NET (GEP-NET), encompassing both traditional gastrointestinal carcinoids and pancreatic endocrine tumors,

Table 2 Hormone production by tumor location[15,9,31,43]

Location	Hormones
Stomach	Histamine, Gastrin, Serotonin, Somatostatin, Gastrin Releasing Peptide
Duodenum/Upper Jejunum	Gastrin, Serotonin, Somatostatin, Gastrin Releasing Peptide
Ileum/Cecum	Enteroglucagon, Serotonin, Substance P, Tachykinins
Appendix	Enteroglucagon, Peptide YY, Serotonin, Somatostatin
Colon/Rectum	Enteroglucagon, Serotonin, Somatostatin
Pancreas	ACTH, Calcitonin, Cholecystokinin, Corticotropin-Releasing Hormone, Gastrin, Glucagon, Growth Hormone-Releasing Hormone, Growth Hormone-Releasing Factor, Insulin, Neurotensin, Pancreatic Polypeptide, Parathyroid Hormone-Related Peptide, Prolactin, Somatostatin, Vasoactive Intestinal Peptide

are replacing the less descriptive and often times pathologically and clinically more confusing term "carcinoid"[3,9]. In 2000, the World Health Organization (WHO) classification replaced "carcinoid" with the terms neuroendocrine tumors and neuroendocrine carcinomas to describe gastrointestinal neoplasms originating from the diffuse system of neuroendocrine cells[9]. Along with developing tumor node metastasis staging and grading systems[10-14], the WHO classification[9] provides an improved system for determining prognosis and treatment and includes three main groups subdivided by organ of tumor origin: (1) well differentiated neuroendocrine tumors (benign behavior or uncertain malignant potential-"carcinoids"); (2) well differentiated neuroendocrine carcinomas (low-grade malignancy-"malignant carcinoids"); and (3) poorly-differentiated carcinomas (high-grade malignancy). This classification replaces the previous outdated system which was based on embryologic cell of origin (foregut, midgut, hindgut) and shared little correlation between tumor behavior and tumor location especially for neoplasms originating in the foregut (tracheobronchopulmonary, gastric, and pancreatic tumors)[3,9]. Histologically, tumor proliferation capacity is measured by Ki-67 staining with Ki-67 Index < 2% seen in grade Ⅰ tumors, 2%-20% in grade Ⅱ tumors, and > 20% tumor cell involvement in grade Ⅲ GEP-NET[11].

Cells originating from the diffuse system of neuroendocrine cells within the gastrointestinal tract share phenotypic similarities with neural cells in their expression of synaptophysin, NSE, and chromogranin A[3,10]. Useful as GEP-NET markers found on the secretory vesicles of neuroendocrine cells, these proteins usually remain independent of cellular production of hormones that are stored within the vesicles[3,10,15]. Hormone production and biologic activity generally varies by GEP-NET location (Table 2) and less than half of the known hormones originating from at least 15 different types of endocrine cells are expressed by GEP-NET[15]. Many tumors remain clinically silent and may present with intestinal obstruction as a result of tumor-induced fibrosis rather than signs or symptoms of secretory products[3]. The classic carcinoid syndrome (cutaneous flushing and secretory diarrhea) occurs in less than 10% of patients[3] and typically in the setting of hepatic metastases.

Diagnostic evaluation

Initial evaluation of patients with a suspected GEP-NET should include a serum chromogranin A level[3,16]. Elevated in approximately 80% of patients with neuroendocrine tumors regardless of location and functional activity, chromogranin A levels also appear to correlate with overall tumor burden[17]. Twenty-four-hour urinary 5-HIAA levels as well as serum gastrin, histamine, serotonin, and substance P levels should be included as part of the initial evaluation when the presentation is consistent with carcinoid syndrome[3]. Urinary 5-HIAA elevation sensitivity is as high as 100% with a specificity of 88% for the carcinoid syndrome[18]. Care must be taken to avoid medications and foods that may affect urinary 5-HIAA excretion; large amounts of serotonin are in foods as avocados, bananas, eggplant, kiwi, pineapple, plums, tomatoes, and walnuts and may cause false positive results[16].

Patients with positive biochemical markers should be evaluated with somatostatin receptor scintigraphy (^{111}Indium-labeled octreotide scan) for tumor localization as well as either CT or magnetic resonance imaging (MRI) to identify mass lesions, mesenteric fibrosis, and lymphadenopathy[3,16]. ^{111}Indium-labeled octreotide scan is useful in detection of both primary and metastatic tumors with sensitivity as high as 90%[3,19]. CT and MRI play an important role in identification of primary tumors and metastatic disease; however, they may underestimate the extent of disease in up to 25% of cases[20,21] and overall sensitivities around 80% are lower than ^{111}Indium-labeled octreotide scanning[3]. Radiolabeled metaiodobenzylguanide (^{123}I-MIBG) scanning may be used in patients on long-acting octreotide medications which interfere with somatostatin receptor scintigraphy[3]. Radiolabeled 5-HTP positron emission tomography has demonstrated better sensitivities than CT and octreotide scanning; however, it is not widely available and is generally still considered an investigational modality[3,21,22]. Barium studies, including small-bowel-follow-through, play little if any role in tumor localization with the availability of other diagnostic modalities with increased sensitivity[23].

Following tumor localization, biopsy for tissue diagnosis should be obtained including performing upper endoscopy and colonoscopy with ileoscopy as clinically indicated[3,16]. Small bowel enteroscopy has low diagnostic sensitivities as well as a limited ability to evaluate the distal jejunum and ileum and has largely been replaced by capsule endoscopy in both diagnostic and surveillance roles[3,24,25]. Endoscopic ultrasound (EUS) plays in

important role in guiding management as it is accurate in assessing tumor size and depth of invasion especially in gastric, duodenal, and rectal carcinoid tumors[26,27].

Site specific information

Gastric carcinoids are typically divided into Type Ⅰ, Ⅱ, and Ⅲ tumors with some classifications including Type Ⅳ tumors[9]. Type I and Ⅱ gastric carcinoid tumors develop in response to hypergastrinemia effects on enterochromaffin-like cells of the oxyntic mucosa found in the gastric fundus and body[28-30]. Type I are the most common gastric NET tumors usually presenting as small multifocal lesions associated with autoimmune chronic atrophic gastritis and hypergastrinemia in the setting of low gastric acid output[3,9,30]. They have an excellent prognosis with 5-year survival rates > 95%[3]. Type Ⅱ gastric NET develop in patients with Multiple Endocrine Neoplasia type-1 (MEN-1) associated Zollinger-Ellison syndrome (ZES) as a result of tumor driven hypergastrinemia in the setting of an autosomal dominant mutation of the *MEN-1* gene located on chromosome 11q13[9,29,30]. Type Ⅱ gastric NET rarely develop in patients with sporadic ZES[29]. Prognosis is good with 5-year survival rates of 70%-90%[3].

Type I and Ⅱ tumors < 1 cm in size without extension into the muscularis propria on EUS can initially be managed with endoscopic mucosal resection[3,26,31]. When more than 5 lesions are present, tumor size is > 1 cm, or recurrence occurs at a site of previous endoscopic resection, local surgical excision is recommended[3]. Type Ⅱ lesions may require aggressive gastrectomy as well as surgical resection of the underlying gastrinoma[3]. Surveillance endoscopy with biopsy should be performed every six months following both endoscopic and surgical tumor removal[3].

Type Ⅲ tumors are sporadic gastric carcinoids which develop in normal gastric mucosa in the setting of normal gastrin levels[3,9]. They are aggressive with deep invasion and the potential for metastatic disease characteristic of even small primary tumors[26]. Five-year survival rates are < 35%[3]. Type Ⅳ tumors are neuroendocrine carcinomas which are indistinguishable from gastric adenocarcinomas with the exception of the presence of neuroendocrine cells within the tumor matrix[3]. Both type Ⅲ and Ⅳ tumors should be managed surgically with complete or partial gastrectomy[3,9].

Small intestine

Duodenal: Five types of duodenal neuroendocrine tumors have been described[32]: (1) gastrinomas which may occur sporadically or in the setting of MEN-1/ZES and are the most common duodenal NET[3,9,32]; (2) somatostatinomas which usually occur in the ampullary/periampullary region and are more likely to be associated with von Recklinghausen's disease (neurofibromatosis type 1)[3,33]; (3) gangliocytic paraganglionomas[3,9,32]; (4) nonfunctioning NET which contain serotonin-, gastrin-, or calcitonin-positive cells[3,9]; and (5) neuroendocrine carcinomas[3,32,33]. Overall 5-year survival for duodenal carcinoid lesions is 60%[3]. Endoscopic resection may be considered for nonmetastatic duodenal (and ampullary) lesions measuring up to 2 cm if the tumor is confined to the mucosa and submucosa on EUS examination[3,33-35]. Surgical resection should be performed on tumors > 2 cm[34,35]. While distant metastases rarely occurs with duodenal NET, lymph node metastases may occur in tumors < 1 cm and surgical resection should be performed in all patients with evidence of lymph node involvement on pretreatment imaging studies[35].

Jejuno-Ileal: Terminal ileum NET are the most common GEP-NET. They are frequently found at an advanced stage with metastatic disease to the liver present in 50% and regional lymph node involvement in up to 70% of patients regardless of primary tumor size[21]. Associated mesenteric fibrosis, nodal metastases, and desmoplastic reactions involving mesenteric vessels may lead to nonspecific abdominal pain, gastrointestinal bleeding, intermittent ischemia, or bowel obstruction. These symptoms may prompt emergent surgical intervention and subsequent diagnosis of a previously unidentified jejunal or ileal NET in up to 40% of patients[3,21]. Ileal NET are associated with the carcinoid syndrome in the setting of liver metastases in approximately 20% of cases[9,21]. While the 5-year survival rate is 60% for both jejunal and ileal tumors, it is as low as 18% when hepatic metastases are present[3]. Surgical resection of the primary tumor as well as *en bloc* resection of regional lymph nodes is recommended and should be performed even when hepatic metastases is present in order to delay progression and local complications of disease[21,31].

Appendix: Appendiceal NET are the most common appendiceal tumor[21]. They are often found incidentally during appendectomy with the majority (90%) of tumors < 1 cm in size. Overall 5-year survival for appendiceal NETs is 98% for benign tumors and 27% for malignant tumors[3]. Metastatic disease rarely occurs with tumors < 2 cm and the occurrence of metastases increases with increasing tumor size over 2 cm[3,21,36]. Tumors > 2 cm should be managed with right hemicolectomy. Appendectomy should be performed in tumors < 2 cm in size with right hemicolectomy considered for tumors 1-2 cm based on pathologic criteria (invasion into mesoappendix, serosal or lymphovascular invasion, involvement of tumor margins, positive lymph nodes, or Ki67 index > 2% on immunohistochemistry staining)[21]. Variant mixed endocrine/exocrine goblet-cell (adenocarcinoid) tumors are more aggressive lesions associated with a poorer prognosis and higher rates of both metastatic and recurrent disease and should be managed with right hemicolectomy regardless of tumor size[21,36].

Colon: Neuroendocrine tumors rarely occur in the colon with many previously reported cecal NET representing appendiceal tumors[3,9]. Clinical presentation of colonic NET includes change in bowel habits, gastrointestinal

bleeding, abdominal pain, weight loss, and asymptomatic lesions found during screening colonoscopy is generally indistinguishable from other mass lesions of the colon[3,9]. Most tumors are > 2 cm in size with invasion into the muscularis propria at the time of diagnosis and overall prognosis is poor with 5-year survival rates of only 33%-42%[3]. Wide surgical resection with lymph node dissection is recommended for management of colonic NET[3] as metastatic disease is common at the time of diagnosis[9]. Local excision may be considered for tumors < 2 cm in size[3]; however, data regarding metastatic disease in this setting are limited.

Rectum: Frequently found as small, asymptomatic submucosal tumors during endoscopic evaluation, rectal NET have an excellent overall prognosis with 5-year survival rates of 87%[3,9]. When present, symptoms may include change in bowel habits, gastrointestinal bleeding, anorectal discomfort, and pruritis ani[3]. Submucosal tumors < 1 cm in size account for 80% of rectal carcinoids[3] and can be managed endoscopically in the absence of muscularis invasion or pararectal lymph node metastases on EUS examination[31,37]. Rectal NETs 1-2 cm in size may be managed with wide surgical excision if there is no evidence of muscularis invasion or lymph node metastasis[3]. Low anterior resection or abdominoperineal resection is recommended for tumors > 2 cm as the risk of metastatic disease increases with tumors > 2 cm in size and with invasion of the muscularis propria[3,9].

Medical therapy

Following surgical resection of a GEP-NET, medical therapy may be required for symptom management related to functional tumor syndromes as well as management of progressive metastatic and residual disease[31,38,39]. Patients with symptomatic functional NET should be considered for somatostatin (SST) analog (short- or long-acting octreotide) or interferon- α therapy alone or in combination[3,31,38,39]. In addition to reducing symptoms in patients with carcinoid syndrome[40,41], VIPoma associated Verner-Morrison syndrome (watery diarrhea, hypokalemia, and achlorhydria)[40,42], and glucagonoma associated necrolytic migratory erythema[4], SST analogs may also play a role in growth inhibition of nonfunctioning NET[39,41]. Interferon-α therapy may be considered in patients who become intolerant or resistant to SST analog therapy as it has also been shown to reduce diarrhea and flushing in patients with carcinoid syndrome[39].

Systemic chemotherapy or peptide receptor radionuclide therapy with I-131 MIBG, Yttrium^{90}, or Lutetium^{177} should be considered in patients with metastatic disease with transarterial embolization/chemoembolization or radiofrequency ablation considered when metastases are limited to the liver[3,31,38,39].

Patients undergoing biologic or cytotoxic therapies should have their clinical response to treatment monitored every 3 mo[11]. Biochemical markers (based on the functional status of their underlying tumor) should be followed every 3-6 mo along with CT or MRI scanning every 6 mo for 5 years following curative surgical resection[11].

In a conclusion, GEP-NET are relatively rare neoplasms of the gastrointestinal tract with variable clinical presentation, morbidity, and mortality dependent on tumor location, metastatic potential, and functional biologic status. Staging and classification systems for GEP-NET are likely to continue to evolve along with further development of tumor directed diagnostic and therapeutic modalities as our understanding of GEP-NET continues to expand over time.

COMMENTS

Case characteristics

This case series describes a wide spectrum of benign gastrointestinal neuroendocrine (carcinoid) tumors (NET).

Clinical diagnosis

The diagnosis and management of NET originating from the luminal gastrointestinal tract and will not include pancreatic NET.

Imaging diagnosis

Computed tomography and magnetic resonance imaging play an important role in identification of primary tumors and metastatic disease.

Experiences and lessons

Gastroenteropancreatic NET are relatively rare neoplasms of the gastrointestinal tract with variable clinical presentation, morbidity, and mortality dependent on tumor location, metastatic potential, and functional biologic status.

Peer review

This is a very good example of a case series combined with a good review.

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TOPIC HIGHLIGHT

Jose Manuel Ramia, MD, PhD, FACS, *Series Editor*

Pathological features and diagnosis of intraductal papillary mucinous neoplasm of the pancreas

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Abstract

Intraductal papillary mucinous neoplasm (IPMN) of the pancreas is a noninvasive epithelial neoplasm of mucin-producing cells arising in the main duct (MD) and/or branch ducts (BD) of the pancreas. Involved ducts are dilated and filled with neoplastic papillae and mucus in variable intensity. IPMN lacks ovarian-type stroma, unlike mucinous cystic neoplasm, and is defined as a grossly visible entity ($\geqslant$ 5 mm), unlike pancreatic intraepithelial neoplasm. With the use of high-resolution imaging techniques, very small IPMNs are increasingly being identified. Most IPMNs are solitary and located in the pancreatic head, although 20%-40% are multifocal. Macroscopic classification in MD type, BD type and mixed or combined type reflects biological differences with important prognostic and preoperative clinical management implications. Based on cytoarchitectural atypia, IPMN is classified into low-grade, intermediate-grade and high-grade dysplasia. Based on histological features and mucin (MUC) immunophenotype, IPMNs are classified into gastric, intestinal, pancreatobiliary and oncocytic types. These different phenotypes can be observed together, with the IPMN classified according to the predominant type. Two pathways have been suggested: gastric phenotype corresponds to less aggressive uncommitted cells (MUC1 -, MUC2 -, MUC5AC +, MUC6 +) with the capacity to evolve to intestinal phenotype (intestinal pathway) (MUC1 -, MUC2 +, MUC5AC +, MUC6 - or weak +) or pancreatobiliary /oncocytic phenotypes (pyloropancreatic pathway) (MUC1 +, MUC 2-, MUC5AC +, MUC 6 +) becoming more aggressive. Prognosis of IPMN is excellent but critically worsens when invasive carcinoma arises (about 40% of IPMNs), except in some cases of minimal invasion. The clinical challenge is to establish which IPMNs should be removed because of their higher risk of developing invasive cancer. Once resected, they must be extensively sampled or, much better, submitted in its entirety for microscopic study to completely rule out associated invasive carcinoma.

Key words: Mucinous pancreatic cysts; Intraductal papillary mucinous neoplasm; Main duct intraductal papillary mucinous neoplasm; Branch duct intraductal papillary mucinous neoplasm; Mucins

Core tip: The authors review the main pathological features of intraductal papillary mucinous neoplasm (IPMN) of the pancreas, including diagnostic criteria and relevance of macroscopic (*i.e.*, main duct, branch duct and mixed or combined) and microscopic (*i.e.*, gastric, intestinal, pancreatobiliary and oncocytic) IPMN classification. Different pathways, mucin immunophenotypes and invasive carcinoma related to IPMN are addressed. Differential diagnosis with pancreatic intraepithelial neoplasm, mucinous cystic neoplasm and other mucinous and non-mucinous pancreatic cystic lesions are

also included.

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DEFINITIONS AND INTRODUCTION

Intraductal papillary mucinous neoplasm (IPMN) of the pancreas is a grossly visible, noninvasive epithelial neoplasm of mucin producing cells arising in the main pancreatic duct or its branches. Intraductal growth of neoplastic cells usually forms papillae in a variable extension, although it can rarely be completely flat. Involved ducts are dilated and filled with mucus in variable intensity[1-4]. The mucus produced by the IPMN can protrude through the duodenal papilla and this sign, so-called "fish-eye ampulla", is virtually diagnostic, although it has been observed in only about 25% of cases[5,6]. IPMN lacks ovarian-type stroma, unlike mucinous cystic neoplasm (MCN) of the pancreas[7]. IPMN is defined as a grossly visible entity, unlike pancreatic intraepithelial neoplasm (PanIN), which is defined as a microscopic lesion[8].

IPMN was previously reported under a variety of terms (mucinous producing cancer[9], ductectatic-type mucinous cystadenoma and cystadenocarcinoma[10], diffuse villous adenoma[11] and intraductal papillary neoplasm of the pancreas[12]) that referred to some of the main features of these lesions. Currently, use of these terms is discouraged. Intraepithelial papillary lesions morphologically analogous to IPMNs develop in the biliary tree, including bile ducts[13], gallbladder[14] and ampullary region[15], and are also reported under a variety of terms.

Most IPMNs are diagnosed between 60 and 70 years of age. There is a slightly higher prevalence in men than women[16]. IPMNs are mostly located in the pancreatic head (70%). About 20% are placed in the body-tail and about 5%-10% show diffuse involvement of the gland. Most are solitary lesions but 20%-40% are multifocal[1,17]. IPMNs can reach a large size before diagnosis because of the slow and indolent growth. However, with the use of high-resolution imaging techniques, very small incidental pancreatic cysts, including IPMNs, are increasingly being identified[18,19]. In the Laffan *et al*[19] series, radiological incidental pancreatic cysts were detected with a mean size of 8.9 mm (range 2-38 mm) in 2.6% of adults without known pancreatic disease. It has been suggested that most of them are IPMNs originating from the small branch ducts but pathological data are missing[20]. On the other side, older surgical data, in which IPMN represents 20% of all pancreatic cysts, probably underestimate its prevalence[1].

IPMNs may exhibit different degrees of dysplasia in the epithelium but even those with high-grade dysplasia or carcinoma in situ have a very good prognosis after resection[21-24]. However, about 40% of IPMNs show invasive pancreatic carcinoma at diagnosis[18]. The prognosis of these patients critically worsens when invasive carcinoma arises, although in the case of minimal invasion, the prognosis is not as severe[23,25]. The clinical challenge is to establish which IPMNs can be managed by clinical and radiological follow-up without requiring surgical excision and which should be removed because they are likely to develop invasive cancer. Progress has been made in the preoperative assessment of the risk of malignancy of pancreatic cystic lesions. Currently, there is an international consensus for the preoperative management of these patients based on clinical and radiological criteria, published in 2006 (the so called Sendai criteria) and updated in 2012 (the Fufuoka guidelines)[7,18]. Nevertheless, the preoperative diagnostic accuracy of pancreatic cystic neoplasms is still far from optimal[26,27].

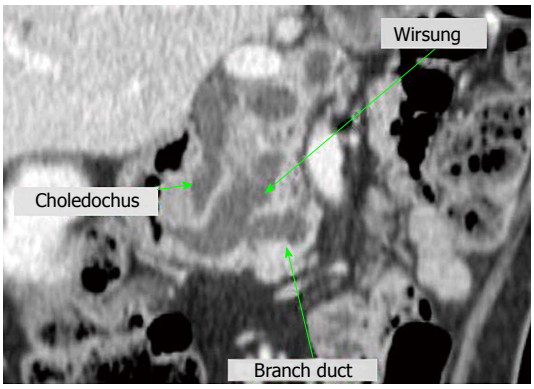

Figure 1 Computerized tomography scan demonstrating massive dilatation of the main pancreatic duct and its branches. Obstructed bile duct is also dilated.

MACROSCOPIC PATHOLOGY

IPMN appears as a dilatation of the main duct or as one or more cysts communicated with the excretory duct system. IPMNs are cystic lesions so the observation of any solid nodule should be suspected of associated invasive carcinoma. However, it should be noted that the invasive carcinoma, especially if small, may be overlooked macroscopically.

DEFINING SIZE OF IPMN

IPMN is defined as a grossly visible lesion and, mostly based on radiological criteria, it is typically considered to be 1 cm or more in size[1,7] (Figure 1). More recently, the Fukuoka guidelines have proposed to reduce the minimum size for radiological diagnosis of IPMN to 5 mm, which increases diagnostic sensitivity without losing specificity. According to this consensus, pancreatic cysts of > 5 mm in diameter that communicate with the main pancreatic duct, especially if there is no pancreatitis, and/or diffuse dilation of the main pancreatic duct of > 5 mm without other causes of obstruction are sufficient

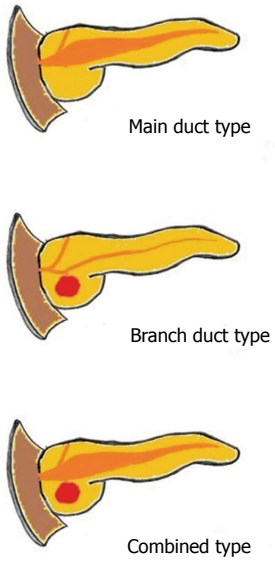

Figure 2 Scheme of macroscopic classification of intraductal papillary mucinous neoplasm.

radiological criteria for IPMN[18].

Main duct, branch duct and mixed or combined types

According to their main location, IPMNs are classified into main duct (MD) type (16% to 36%), branch duct (BD) type (40% to 65%) and mixed or combined type (15% to 23%)[21,23,28,29] (Figure 2). This classification reflects biological differences with important prognostic implications[23,30]. Most MD type and combined type IPMNs exhibit malignancy (*i.e.*, high-grade dysplasia or carcinoma), with about 45% having an associated invasive carcinoma[18,22,31]. In contrast, most BD type show low-grade dysplasia and only about 15% are associated with invasive carcinoma[18]. The natural history of BD type under 30 mm in size and without mural nodules is particularly favorable[5,32]. Currently, this macroscopic classification has a substantial practical impact on the preoperative clinical management based on imaging findings[7,18].

MD type (Figure 3A) is essentially located in the main pancreatic duct[11,18]. At external examination, the pancreas may be thickened in the affected area. After opening, this type typically shows dilatation of the main duct with irregular outline and the lumen is filled by mucus and villous or papillary projections. The rest of the pancreas often shows the appearance of obstructive chronic pancreatitis due to pancreatic duct obstruction[17]. Most of the MD type are located in the pancreatic head but one third of them are in the body and tail, and almost 5% in the entire main pancreatic conduct (diffuse MD type)[22]. Eventually, some cases of MD type are multifocal. However, a particular lesion may be macroscopically from a focal type but microscopically exhibit multifocal or diffuse extension throughout the duct.

BD type (Figure 3B) is located predominantly in secondary branches of the pancreatic ductal system[1,18]. Typically, the affected duct has the appearance of a mucus-filled cyst. As there is no main pancreatic duct obstruction, the remaining pancreas may have normal appearance[17,33]. Most of the IPMN of BD type occurs in the pancreatic head and very commonly in the uncinate process. About 25%-40% are multifocal[18,22].

The mixed or combined type of IPMN (Figure 3C) primarily affects both the main pancreatic duct and secondary branches[1,18]. Hypothetically, combined type of IPMN might result from the progression of MD or BD types or it could be a distinct disease. Clinical and biological characteristics are similar to those of the MD type, so it is thought that combined IPMN is most likely an extension of the MD type to the branch ducts[22,30].

Fistula formation and other additional macroscopic features of IPMNs

Uncommonly, the neoplastic papillae extends out of the ampulla and onto the surface of the periampullar duodenum or into the distal common bile duct[17].

Also infrequently, IPMN can develop a fistula to neighboring organs, among them the duodenum, stomach, choledochus, colon and small intestine. The fistula may be related to benign IPMNs (*i.e.*, low or moderate degree of dysplasia), malignant IPMNs (*i.e.*, high-grade dysplasia) or invasive carcinoma associated with IPMN (often a colloid carcinoma)[34]. Two scenarios can be observed in the pathogenesis of these fistulas: (1) mechanical penetration due to excessive pressure in the mucin filled ducts, in addition to inflammatory stimulation or autodigestion of enzyme rich fluids; and (2) direct invasion, *i.e.*, with presence of invasion of the tissue around the fistula[34,35]. Mechanical penetration can occur regardless of the presence of malignant cells at the surface of the fistula (without direct tissue invasion by carcinoma). In conclusion, the presence of fistulas in IPMN does not necessarily mean malignancy and should not be confused with invasive carcinoma.

In rare cases, IPMN has been described as causing *pseudomyxoma peritonei*[36], for instance by associated acute pancreatitis with fistula formation to the abdominal cavity[37] or after intraoperative manipulation of the pancreas[22].

Extensive *pancreatic calcification* has rarely been described in patients with IPMN. This obstructive calcifying pancreatitis, presumed to be caused by the IPMN, may lead to preoperative diagnostic confusion and delay in the diagnosis of the papillary neoplasia[38,39].

MICROSCOPIC PATHOLOGY

Histologically, IPMN is a heterogeneous group of lesions with different degrees of dysplasia and different cellular phenotypes. The underlying stroma shows a conventional fibrous tissue, which by definition can not be of ovarian type, as seen in MCN[1].

Grades of dysplasia

Based on the degree of cytological atypia and abnormal crowding of the epithelium, IPMN is classified into three categories: IPMN with low-grade dysplasia, IPMN with intermediate-grade dysplasia and IPMN with high-grade

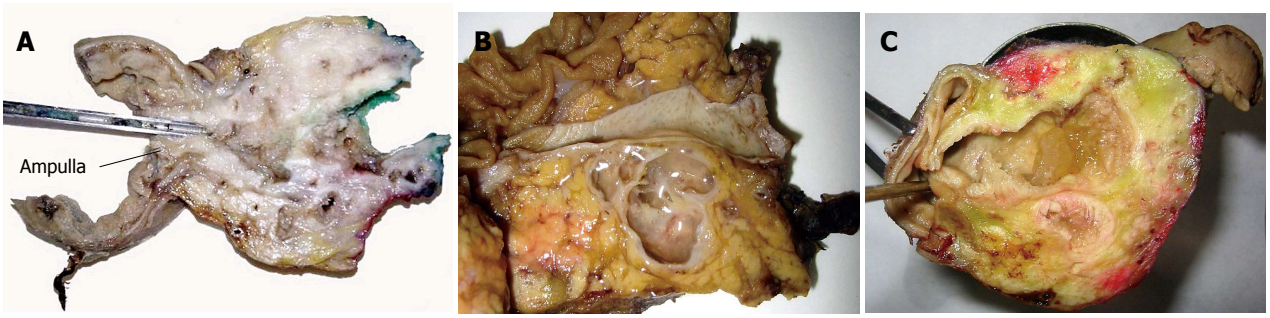

Figure 3 Macroscopic classification of intraductal papillary mucinous neoplasm. A: Main duct type; B: Branch duct type; C: Combined type.

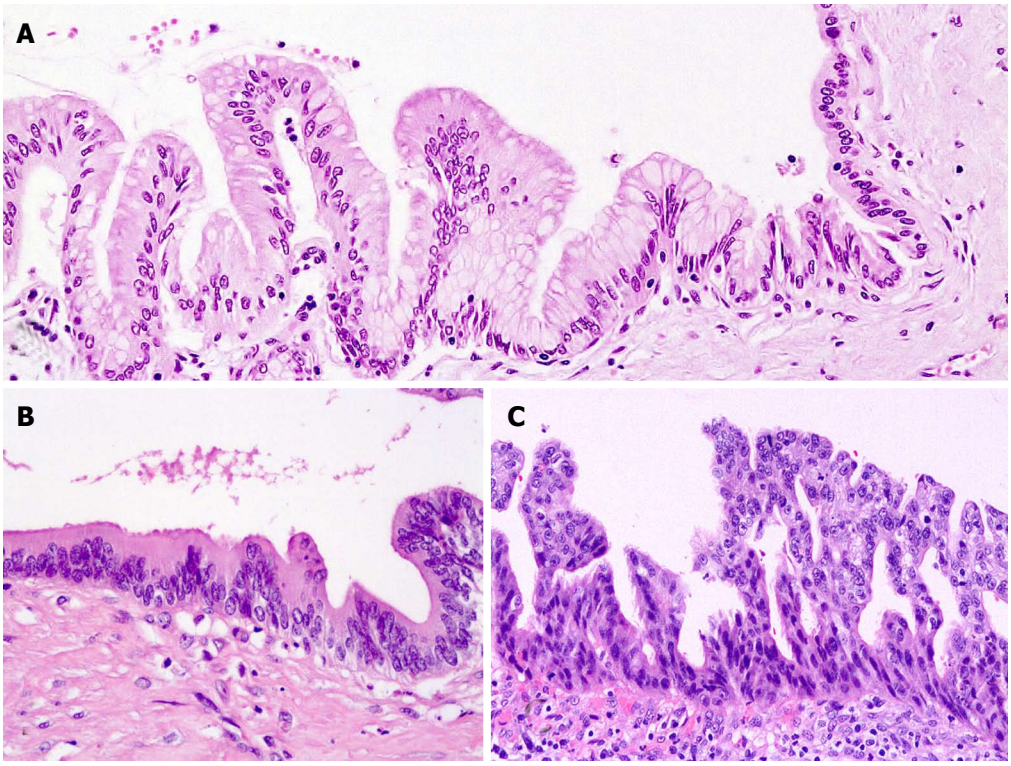

Figure 4 Different degrees of dysplasia. A: Low-grade (gastric IPMN). See transition with non dysplastic normal duct epithelium (right side); B: Intermediate-grade (intestinal IPMN); C: High-grade (pancreatobiliary IPMN). IPMN: Intraductal papillary mucinous neoplasm.

dysplasia (Figure 4). This nomenclature, currently adopted by the WHO system, replaces the terms of adenoma (low grade), borderline (intermediate grade) and carcinoma *in situ* (high grade dysplasia)[1,7]. Low-grade dysplasia is characterized by a uniform monolayer of columnar cells with basal nuclei showing no or minimal atypia. In the intermediate-grade of dysplasia, nuclear atypia is higher, with nuclear pleomorphism, nuclear enlargement and pseudostratification. In high-grade dysplasia, there is marked cytological atypia and complex architecture with cribriform groups and budding of neoplastic cells into the lumen[4,17]. It is common to observe different grades of dysplasia within a given lesion, which suggests the development of dysplasia from a lower to a higher grade. The distinction of dysplasia grade is important, with associated invasive carcinoma commonly immersed in areas of high-grade dysplasia[40,41]. In each individual case, the lesion should be classified according to the highest grade of dysplasia observed[1].

Mucins and microscopic IPMN phenotypes

On the basis of the cytoarchitectural features and immunophenotype, IPMNs are classified into four histopathological types: gastric, intestinal, pancreatobiliary and oncocytic IPMNs[1], accounting for 49%-63%, 18%-36%, 7%-18% and 1%-8% of total cases in two large series[3,42] (Table 1). This classification is not only descriptive but also indicative of different pathways of differentiation and progression to invasive carcinoma[23,43-48]. The above nomenclature prevails over other terms proposed for these lesions[3]. The so called villous dark cell, papillary clear cell and compact cell types respectively correspond to intestinal, gastric and oncocytic cell types[49,50], whereas null cell type corresponds to gastric cell type[51].

Core proteins for mucins (MUCs) can be detected by immunohistochemistry. The mucin expression profile by

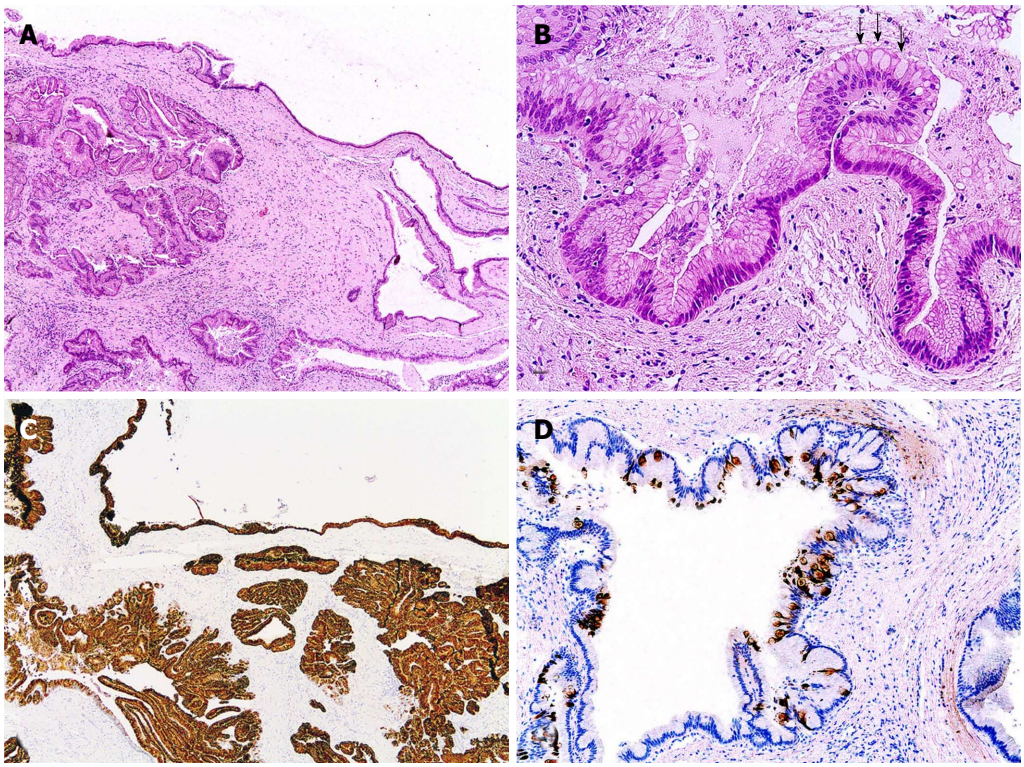

Figure 5 Gastric intraductal papillary mucinous neoplasm. A: The neoplasm involves branch ducts with a multicystic appearance; B: Columnar cells with basal nucleus and apical mucin. Notice the scattered goblet cells (arrows); C: Immunohistochemical MUC5AC expression (colored brown by diaminobenzidine); D: MUC2 highlighting the goblet cells. MUC: Mucin.

Table 1 Mucin immunoprofile in intraductal papillary mucinous neoplasm

Morphological type	MUC1	MUC2	MUC5AC	MUC6
Gastric	-	-[1]	+	+
Intestinal	-	+	+	± weak
Pancreatobiliary	+	-	+	±
Oncocytic	+	-[1]	±[1]	+

[1]Scattered positive goblet cells can be present. MUC: Mucin.

the IPMN cells is a major contributor to their phenotypic classification. Mucins are high molecular weight glycoproteins produced by different types of epithelial cells. Some mucins are normally located in the cell membrane, like MUC1 (also called mammary-type mucin or pan-epithelial membrane mucin), whereas others are normally secretory products, including MUC2 (intestinal type gel forming mucin), MUC5AC (gastric surface mucous epithelial mucin) and MUC6 (gastric pyloric glandular mucin)[52,53].

In normal pancreatic tissue, there is MUC1 expression (limited to centroacinar cells, intercalated and intralobular ducts and focally in the interlobular ducts) and sometimes there is expression of MUC6 (limited to the acini) but MUC2 and MUC5AC are not expressed[50]. In pancreatic neoplasms, MUC1 is considered a marker of aggressiveness, being expressed in some IPMNs, higher-grade cases of PanIN and in conventional (*i.e.*, tubular) ductal adenocarcinoma. On the contrary, MUC2 is considered a marker of a more indolent phenotype, being expressed in some IPMNs and in colloid carcinoma[43].

Gastric type IPMN is frequently observed in BD type. The great majority of gastric IPMNs exhibit only low-grade dysplasia and association with invasive carcinoma is uncommon. It has been observed that, when developing, invasive carcinomas are conventional type with more aggressive characteristics and with a poorer prognosis than those arising from intestinal or pancreatobiliary IPMNs[23,16]. Because the gastric type IPMNs associated with these invasive carcinomas is usually benign (*i.e.*, with only low or intermediate grade of dysplasia), it has been questioned whether in these cases the gastric type IPMN represents the invasive carcinoma or whether it is just its background or coexisting benign IPMN[42]. Gastric type IPMN consists of columnar cells with basal nuclei and abundant apical cytoplasmic mucin, resembling the foveolar gastric epithelium (it is also called gastric foveolar type IPMN). These lesions are often mainly flat or with low papillary pattern, consisting in thick finger-like papillae[3]. Immunoprofile consists of diffuse expression of MUC5AC and MUC6 without expression of MUC1 and MUC2, although scattered MUC2 positive goblet cells can be present into the lesion[16,44,48,50]. Gastric mucins may show a distribution which mimics mucins found in the gastric mucosa, namely, increased expression of MUC5AC in the superficial or papillary areas, simultaneously of MUC6 located in the basal areas[50] (Figure 5).

Intestinal type IPMNs mimics villous adenomas of the colon. They form elongated papillae of columnar cells with enlarged cigar-like nuclei[3]. Diffuse expression

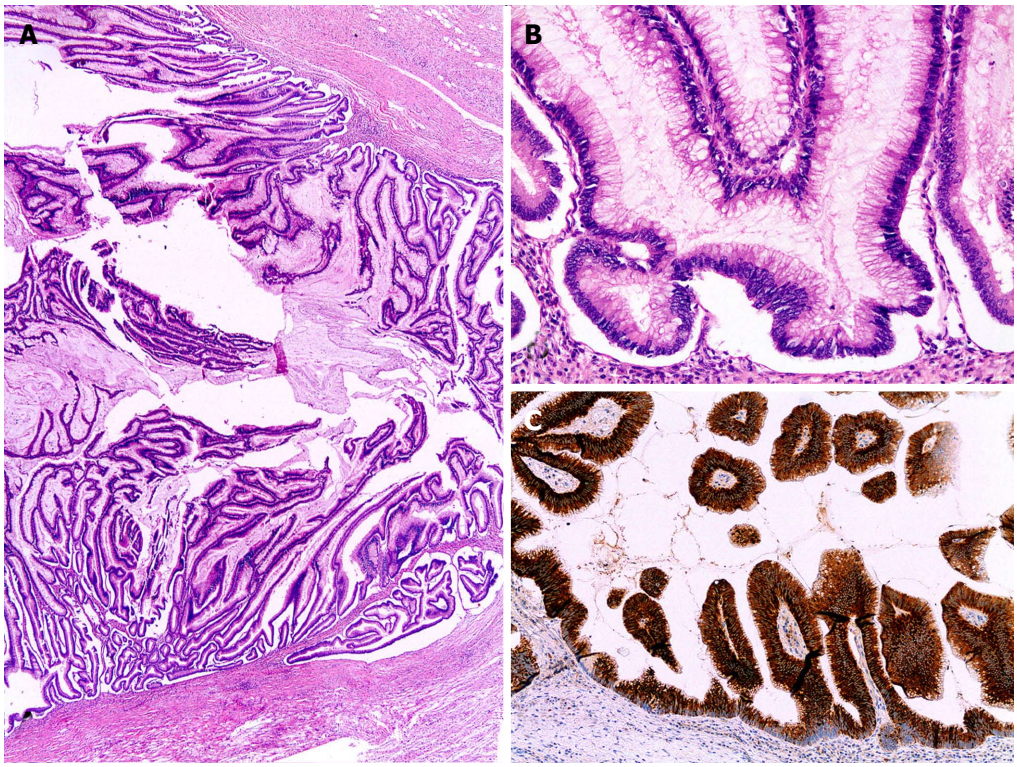

Figure 6 Intestinal intraductal papillary mucinous neoplasm. A: Main duct distended by long papillae; B: Projections of columnar cells with pseudostratified nuclei; C: Immunohistochemical MUC2 expression. MUC: Mucin.

of MUC2 and MUC5AC, weak or negative expression of MUC6 and negativity for MUC1 is the mucin immunoprofile of this type[16,50,48]. In addition, intestinal IPMN exhibits diffuse expression for CDX2, a transcriptional factor related to intestinal differentiation that, like MUC2, has tumor suppressor activity[54]. The intestinal type IPMN frequently exhibits an intermediate or high-grade of dysplasia. It occurs more frequently in the main duct and, when associated with invasive carcinoma, this is often a colloid adenocarcinoma[23,51]. In the absence of invasive carcinoma, intestinal IPMN seems to have a greater potential for long-term recurrence than non-invasive IPMN of other types. Recurrence in the remnant pancreas may be due to multifocality not recognized at the time of surgery or to metachronous development[23] (Figure 6).

Pancreatobiliary type IPMNs usually show high-grade dysplasia and is likely to have a strong predisposition to develop invasive carcinoma[23]. Associated invasive carcinomas usually are of conventional type[23,51]. Pancreatobiliary type IPMN consists of more cuboidal cells with rounded nuclei, often with prominent nucleoli. The neoplastic cells are organized into thin complex and branching papillae with bridging and cribiform patterns[3]. The neoplastic cells express MUC1 and MUC5AC, sometimes MUC6, and are negative for MUC2[3,48] (Figure 7).

Oncocytic type IPMNs (also known as intraductal oncocytic papillary neoplasm) are characterized by neoplastic cells with abundant granular eosinophilic cytoplasm (due to the presence of numerous mitochondria) and also intracellular mucin[1,55]. Most of them have high-grade dysplasia, with complex thick papillae and cribriform structures. The neoplastic cells express MUC1 and MUC6. Expression of MUC5AC is controversial, being constantly present according to some authors but limited to scattered goblet cells according to others. The scattered goblet cells also express MUC2[48,56,57]. Invasive carcinoma associated with oncocytic type IPMN often conserves the oncocytic features[23,42]. An association between the oncocytic type IPMN and minimally invasive carcinoma has been observed[23].

Different pathways in IPMNs

In some cases, different phenotypes can be observed in the same lesion. Each lesion should be classified according to the predominant phenotype, although all the present phenotypes should be recorded[3]. The most common coexistences are gastric with intestinal or gastric with pancreatobiliary type[42]. On the contrary, it is very rare to observe intestinal and pancreatobiliary types together[16]. Oncocytic type has been observed to be associated with gastric and pancreatobiliary types[56]. It has been suggested that the gastric phenotype corresponds to less aggressive uncommitted cells with the capacity to evolve to intestinal phenotype (intestinal pathway) or pancreatobiliary/oncocytic phenotypes (pyloropancreatic pathway) becoming more aggressive[44,48,51]. Gastric foveolar epithelium-like cells (also called null cell type cells) similar to cells of the gastric papillary areas of IPMNs can usually be observed lining the nonpapillary cystic areas of different IPMNs[51]. Pancreatic duct glands are blind-ending outpouches of major ducts with a possible role in epithelial renewal and repair. Epithelium of these glands

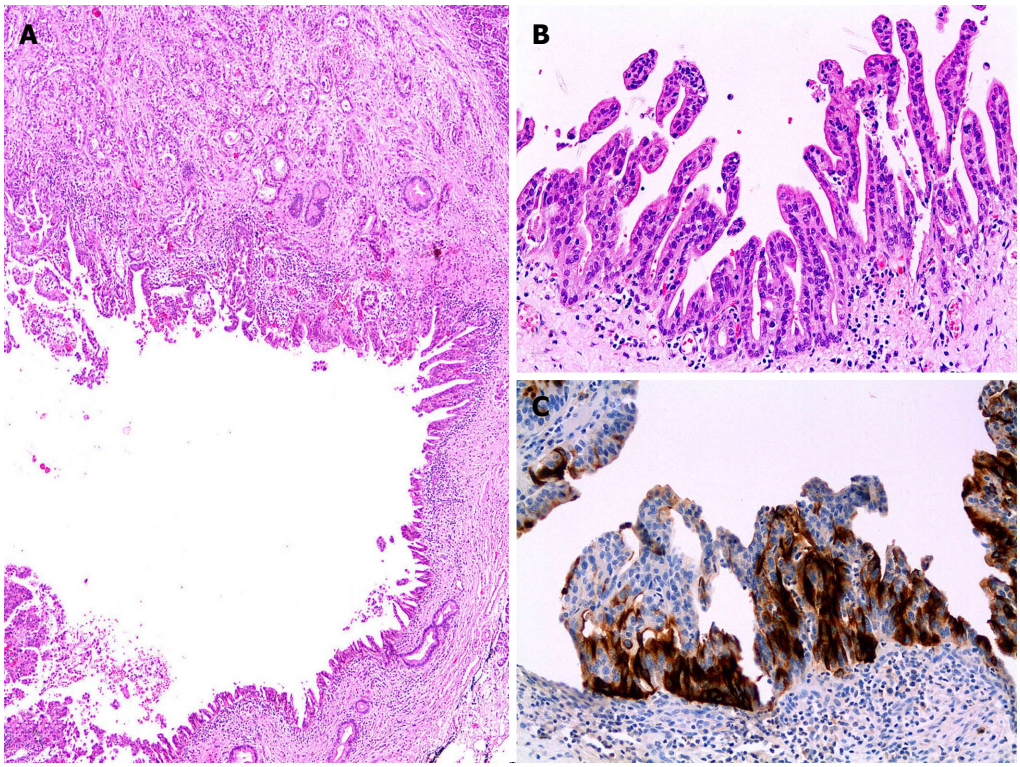

Figure 7 Pancreatobiliary intraductal papillary mucinous neoplasm. A: Intraductal papillary mucinous neoplasm with associated conventional duct carcinoma (upper side); B: Small thin papillae with cuboidal neoplastic epithelium; C: Immunohistochemical MUC1 expression. MUC: Mucin.

is a specialized compartment with production of gastric type mucin (MUC6+) in its normal state. When chronically injured, it becomes hyperplastic and it acquires de novo expression of MUC5AC. It has been speculated that these pancreatic duct glands are a source of gastric mucinous metaplasia and could be the origin of PanIN[58] and IPMNs[6], in addition to its possible role in regeneration and protection of the major ducts.

Intraoperative microscopic assessment of pancreatic margin

Frozen study of pancreatic cut surface during resection of IPMN is accurate to evaluate the completeness of resection. The accuracy of frozen study averages 95%[59]. If invasive carcinoma or high-grade dysplasia is seen in the pancreatic margin, this should be extended. Further resection in cases with lesser degrees of dysplasia in the margin is controversial[18] but may be considered in some cases, depending on the patient's age and the macroscopic type IPMN among other factors[60,61]. Recurrences after resection of non-invasive IPMN with free margin may occur and can be attributed to multifocality[60].

The distinction between IPMN and PanIN may be almost impossible in some cases, although this distinction is not considered crucial in assessing the margin, with the distinction of the degree of dysplasia being most relevant[18]. Those wishing to obtain a pancreatic margin without any degree of dysplasia should be aware that intraoperative differential diagnosis of low-grade dysplasia *vs* non dysplastic epithelium with reactive changes can be impossible to achieve. This should be considered to avoid unnecessary or useless resections. The presence of mucus or duct dilatation at the cut surface does not indicate any additional necessity for resection. De-epithelization of the pancreatic duct margin has been observed to be a prognostic factor for recurrence by some authors and thus they have proposed to do additional resection in cases of eroded epithelium[60].

IPMN AND INVASIVE CARCINOMA

Adenocarcinoma derived from vs concomitant with IPMN

About 40% of IPMNs are associated with invasive pancreatic carcinoma, although the reported risks of malignancy are quite population-dependent and vary considerably (with range between 1.4% and 80.8%)[18,29]. Invasive carcinoma can be uni- or multifocal and occurs most often in MD and combined type than in BD type[18,22]. In patients with IPMN, the distinction must be made between adenocarcinoma derived from IPMN and adenocarcinoma concomitant with IPMN[62]. The first evidently develops from IPMN, while the latter occurs in the pancreas with IPMN but in another location of the organ, therefore without an obvious topological relationship and in the absence of histological transition between the two lesions. Sometimes, the possible relationship between IPMN and invasive carcinoma remains undetermined. In a large series of patients with IPMNs and associated adenocarcinoma, 66%, 17% and 16% corresponded to adenocarcinoma derived from IPMN, concomitant with IPMN, and undetermined, re-

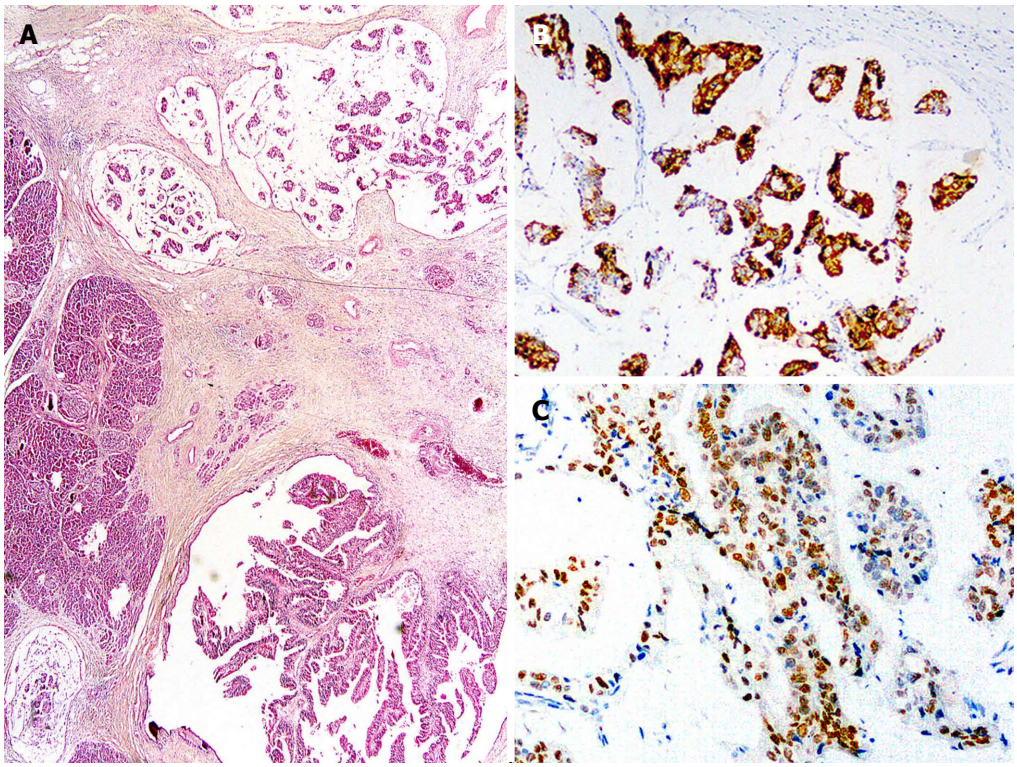

Figure 8 Colloid carcinoma. A: Invasive neoplastic cells floating in pools of mucin (upper side) and associated with intraductal papillary mucinous neoplasm (lower side); B: Immunohistochemical MUC2 expression; C: CDX2 nuclear immunoexpression. MUC: Mucin.

spectively[63]. Among the general population with IPMN, the risk of developing invasive pancreatic carcinoma separately from IPMN was estimated to be 2.8% in a recent cohort analysis[29].

Principal histological types of invasive carcinoma related to IPMN

Most invasive carcinomas related to IPMNs are colloid (mucinous noncystic) carcinomas and conventional (tubular) carcinomas. Mixed colloid-tubular carcinomas or adenocarcinomas with focal colloid features also occur[17,23,24, 29,63-65]. Colloid carcinoma of the pancreas is very commonly associated with IPMN[51]. In fact, some authors argue that it virtually never exists without an associated IPMN (whose detection would depend on the extent of the tumor sampling)[65]. Most IPMNs related to colloid carcinoma are intestinal type[23]. Like intestinal type of IPMN, colloid carcinoma shows diffuse expression of CDX2 and MUC2 (*i.e.*, features of intestinal differentiation)[51] (Figure 8). Prognosis of colloid carcinoma is considered better than conventional ductal carcinoma[18,66]. Conventional invasive adenocarcinoma related to IPMN is most often associated with pancreatobiliary type IPMN (Figure 7A). Both share the more aggressive immunohistochemical profile consisting of MUC1 expression and lack of MUC2 and CDX2 expression[23,51].

Prognosis aspects linked to the existence of invasive carcinoma in IPMN

In general, the prognosis of patients with invasive carcinoma associated with IPMN is better than that of patients with ordinary pancreatic adenocarcinoma (*i.e.*, patients without IPMN), but when matched by disease stage, no prognostic differences appear to exist between the groups, except at an early stage. Best overall prognosis of these patients may lie in their greater frequency of early stage cases and a higher prevalence of colloid carcinomas[21,41,67,68]. A lower frequency of other adverse histological features (*i.e.*, vascular invasion, perineural invasion, involvement of surgical margins and poor tumor differentiation) contribute to better prognosis of IPMN-associated invasive carcinomas[69].

Some authors have observed that invasive carcinoma has a better prognosis if depth invasion is limited[23,25,70-72]. This so called minimally invasive carcinoma has been defined as tumor with slight invasion beyond the pancreatic duct wall[23,70,71] or as carcinoma with infiltration depth up to 5 mm[25,72]. Fukuoka guidelines recommend avoiding use of the term "minimally invasive" because of its variable definition. Instead, it is proposed to substage the category T1 into T1a if carcinoma infiltrates up to 0.5 cm, T1b if > 0.5 cm up to 1 cm, and T1c if infiltrates between 1 and 2 cm[18]. Minimal invasion is more frequently observed in intestinal and oncocytic types of IPMNs than in gastric and pancreatobiliary types[23,72]. Frequently, minimally invasive carcinoma related to IPMN is colloid type[63].

Some patients with resected IPMN without associated invasive carcinoma subsequently develop local invasive carcinoma or metastatic lesions. Multifocal disease with synchronous or metachronous development of tumor in the remnant unresected pancreas may explain the origin

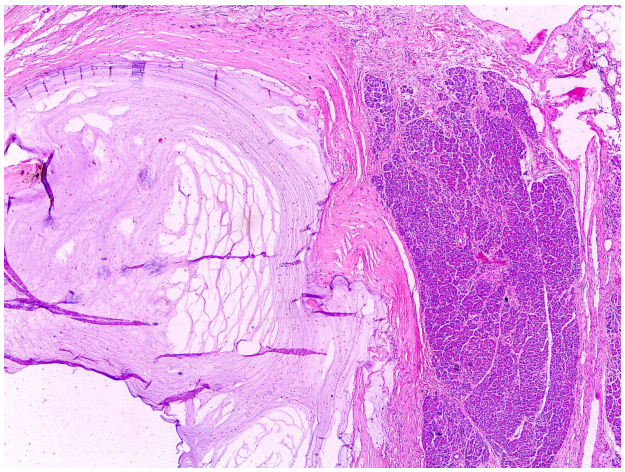

Figure 9 Pseudoinvasion. Mucin spillage dissecting into the pancreatic stroma without neoplastic cells.

of some of these recurrences[24]. In other cases, it is possible that a preexisting small focus of invasive carcinoma passed unnoticed in the pathological study. Because of its critical prognostic significance, a major objective for the pathologist is to rule out the presence of invasion. Initial sampling of the surgical specimen should include all nodular areas because of its higher suspicion of malignancy. As invasive carcinoma may be overlooked by gross examination (especially if it is of small size), the tumor must be extensively sampled or, much better, submitted in its entirety for microscopic study. Measuring the size of invasive carcinoma irrespective of the size of IPMN is required for appropriate staging. If multiple invasive foci exist, they must be measured separately, highlighting the size of the largest focus.

Invasion vs pseudoinvasion

There is usually little difficulty in recognizing invasive carcinoma associated with IPMN when tumor cells are observed penetrating the tissue with a classic infiltrative growth pattern. However, like in other mucin-secreting tumors, IPMNs can exhibit tumor growth by duct expansion (expansive growth) as well as mucous rupture or mucin spillage into the stroma, whose interpretation is controversial[25,73]. Lakes of mucin in the stroma may correspond to colloid carcinoma but also may be due to rupture of a mucus filled duct, presumably by the high intraluminal pressure produced by the mucus itself. IPMN desquamated cells could be transported to the stroma by the extruded mucin, completely simulating colloid carcinoma. Acellular mucin extruded into stroma is not considered invasive cancer (Figure 9). In contrast, mucin spillage containing neoplastic cells is better considered invasive carcinoma[1,17]. On another issue, IPMNs should not be confused with the rarest pancreatic adenocarcinomas with cystic papillary pattern, consisting of large caliber malignant glands with intraluminal papillary structures and pools of intraluminal mucin that mimic noninvasive cystic neoplasms. Elastin stains are very helpful for distinction: unlike normal pancreatic ducts and ducts with IPMN that typically are surrounded by a layer of elastin fibers, there are no elastin fibers around these large invasive malignant glands[74].

DIFFERENTIAL DIAGNOSIS

Other cystic and/or papillary lesions

A major preoperative (*i.e.*, mainly clinical and radiological) differential diagnosis of IPMNs includes neoplastic and non-neoplastic pancreatic cysts: serous cytadenoma (in particular the oligocystic or macrocystic variant)[75], MCNs, solid pseudopapillary neoplasm[76], retention cyst[4], pseudocyst and other less common or clinically relevant entities[20]. In addition, usually solid pancreatic lesions that occasionally are dominated by a cystic, papillary or papillocystic pattern must be considered in this differential diagnosis: acinar cell carcinoma[77], pancreatic endocrine tumors[78] and pancreatic duct adenocarcinoma[74]. In surgical specimens, histological findings usually allow solving the above cited differential diagnosis, sometimes with the assistance of immunohistochemistry. In preoperative management, cyst fluid or pancreatic juice cytology can increase clinical and radiological accuracy diagnoses in pancreatic cysts regarding the distinction between mucinous and non mucinous lineage and malignancy identification. Mucinous cyst cytology cannot accurately discriminate between IPMN and MCN and, although cytology shows high specificity for detecting malignancy, sensitivity is low[79]. Sensitivity for malignancy detection increases if cases with cytological diagnosis of high grade atypia are included, but this reduces the specificity[80,81]. Carcinoembryonic antigen (CEA) pancreatic cyst fluid level may contribute to distinction between mucinous (high CEA levels) and non mucinous (low or no CEA levels), but does not differentiate between benign and malignant. Some authors warn about IPMN dissemination after puncture because of the potential risk of leakage of cyst content. Currently, Fukouka guidelines consider cytological study of mucinous-like cystic lesions in general limited to research, except in centers with expertise in endoscopic ultrasound - fine needle aspiration (EUS-FNA) and cytological interpretation where cytological analysis is recommended for the evaluation of small BD-IPMNs without worrisome features[18]. EUS-FNA with cyst fluid CEA determination may also be required for the differentiation between BD-IPMN and oligocystic serous cystic neoplasm[18,75].

MCN and other mucinous cysts

Focusing on the mucinous category, accurate distinction is not always possible between various mucinous cystic lesions. In a large series of resected mucin-producing neoplasms of the pancreas, 6% of mucinous cystic lesions were undetermined[22]. BD type of IPMN, especially when largely flat, can be confused with MCN if the topological branch ducts relationship is not clear. In addition, although MCN lacks a connection to the duct system, it rarely can fistulize into the ducts and very rarely exhibits intracystic papillary-like growth that may be confused

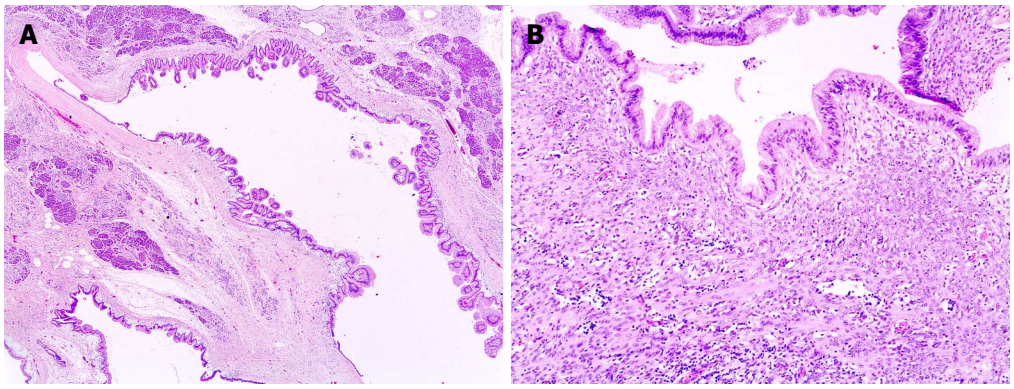

Figure 10 Mucinous cystic neoplasm. A: An example with papillary projections and surrounded by a thick collagenized band; B: Demonstration of ovarian-type stroma, at least focally, leads to diagnosis.

with papillary structures of MD-IPMN[82] (Figure 10A). Ovarian-type stroma in MCN facilitates this differential diagnosis and accentuates the clinical distinction of MCN *vs* IPMN[7] (Figure 10B). MCN occurs in patients usually younger than BD type of IPMN (44.5 years *vs* 66 years in a large series[22]) and it is almost always a single lesion located in the pancreatic body/tail in women, whereas this type of IPMN occurs more commonly in the pancreatic head, can be single or multifocal, and occurs slightly more often in men[7,22]. Cystic mucin-producing pancreatic neoplasms without either IPMN histological features or ovarian-type stroma are better termed indeterminate mucin-producing neoplasms[7]. If such indeterminate cystic lesions exhibit simple mucinous epithelium without cytological atypia, they still are considered neoplasms by some authors[64]. Alternatively, they are termed non neoplastic (or non dysplastic) cystic mucinous lesions by others[83], although it is unclear whether they could represent the earliest manifestation of mucinous neoplasms. Retention cysts should also be considered. They happen because of pancreatic duct obstruction. They usually are unilocular and lined by normal or flattened ductal epithelium without atypia, but sometimes they are described with slight papillary or mucinous change[4]. Therefore, there are no specific limits for the distinction between retention cyst, non neoplastic mucinous cyst and some neoplastic mucinous cysts.

PanIN

PanIN is the other main premalignant lesion of the pancreas besides IPMN. Lesions of PanIN can be flat, micropapillary or papillary, but unlike IPMN which is macroscopically visible, PanIN is defined as a microscopic entity[8]. Although PanIN lesions typically arise in the smaller ducts, it may involve large ducts. In addition, IPMN often extends from larger ducts to smaller pancreatic ducts[4]. The histological distinction between IPMN and PanIN is not always possible. The main issue concerns BD gastric type of IPMN because of its peripheral location and more similar cytohistological appearance and immunohistochemical profile (MUC2 negative and MUC5AC positive)[43,61]. It has generally been assumed that PanIN measures less than 0.5 cm and IPMN over 1 cm. It has been suggested to use a descriptive diagnosis, such as intraductal proliferative lesion of undetermined type, for an especially gray area of 0.5-1 cm featureless diameter[61].

Intraductal tubulopapillary neoplasm

Intraductal tubulopapillary neoplasm (ITPN) is a rare lesion characterized by a more solid intraductal growth without visible mucin secretions and with less cystic aspect than IPMN. Histologically, ITPN is characterized by a complex proliferation of tubules and variable extension of papillary architecture. Neoplastic cells show scant cytoplasmic mucin and uniform high grade dysplasia. Solid areas and necrotic foci are frequently seen. Associated invasive carcinoma is frequently scarce and observed in about 40% of cases[1,84]. ITPN is considered within the spectrum of IPMNs by some authors, although it is regarded as a separate entity by the current WHO system[16].

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TOPIC HIGHLIGHT

Jose Manuel Ramia, MD, PhD, FACS, *Series Editor*

Considerations on pancreatic exocrine function after pancreaticoduodenectomy

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Abstract

The pancreaticoduodenectomy (PD) procedure may lead to pancreatic exocrine and endocrine insufficiency. There are several types of reconstruction for this kind of operation. Pancreaticogastrostomy (PG) was introduced to reduce the rate of postoperative pancreatic fistula. Although some randomized control trials have shown no differences regarding pancreatic leakage between PG and pancreaticojejunostomy (PJ), recently some reports reveal benefits from the PG over the PJ. Some surgeons concern about the performing of the PG and inactivation of pancreatic enzymes being in contact with the gastric juice, and the detrimental results over the exocrine pancreatic function. The pancreatic exocrine function can be measured with direct and indirect tests. Direct tests have the highest sensitivity and specificity for detection of exocrine insufficiency but require tube placement. Among the tubeless indirect tests, the van de Kamer stool fat analysis remains the standard to diagnose fat malabsorption. The patient compliance and time consuming makes it not so suitable for its clinical use. Fecal immunoreactive elastase test is employed for screening of exocrine insufficiency, is not cumbersome, and has been used to study pancreatic function after resection. We analyze the FE1 levels in our patients after the PD with two types of reconstruction, PG and PJ, and we discuss some considerations about the pancreaticointestinal drainage method after pancreaticoduodenectomy.

Key words: Pancreaticoduodenectomy; Pancreaticogastrostomy; Pancreaticojejunostomy; Pancreatic exocrine function; Fecal elastase

Core tip: Many patients present pancreatic exocrine insufficiency after pancreatic resection. Exocrine insufficiency leads to steatorrhoea, flatulence, abdominal pain, weight loss and malnutrition. Extent of resection will determine the severity of insufficiency, but also changes in anatomy may be determining factors. Pancreatogastrostomy is deemed detrimental over the pancreatic function because of the hypothetical inactivation of pancreatic enzymes due to the acid juice of the stomach. In this review we discuss the physiological aspects of the changes in exocrine pancreatic function focusing on the pancreatoenterostomy after a pancreaticoduodenectomy.

Morera-Ocon FJ, Sabater-Orti L, Muñoz-Forner E, Pérez-Griera J, Ortega-Serrano J. Considerations on pancreatic exocrine function after pancreaticoduodenectomy. *World J Gastrointest Oncol* 2014; 6(9): 325-329 Available from: URL: http://www.wjgnet.com/1948-5204/full/v6/i9/325.htm DOI: http://dx.doi.org/10.4251/wjgo.v6.i9.325

INTRODUCTION

After a pancreaticoduodenectomy (PD) procedure the patient has an altered upper gastrointestinal and pancreatic anatomy with potential pancreatic exocrine and endocrine insufficiency. The first known pancreaticoduodenectomy was performed by Alessandro Codivilla on february 1898 in Italy[1], followed by Kausch[2] 11 years later. There are two operation techniques performed predominantly: the classic Whipple operation, developed and modified by Whipple[3], and the pylorus-preserving procedure inaugurated by Watson[4] and popularized by Traverso *et al*[5]. In the classic PD, an antrectomy or distal gastrectomy is associated to the resected specimen. Gastric resection is avoided in the pylorus-preserving modification. Mortality and morbidity are not significantly different between both techniques[6], but the pylorus-preserving procedure improve postoperative weight gain[7-9].

The majority of authors consider pancreatic anastomotic leakage as the primary cause of morbidity and mortality after PD[10-13]. Pancreaticogastrostomy (PG) was introduced to reduce pancreatic leakage. Waugh and Clagett at the Mayo Clinic were the first to use this anastomosis in the clinical setting. Mackie *et al*[14] from the University of Pennsylvania reported their experience and observed lower operative mortality in their institution with implantation of PG. Initially infrequently used, PG is becoming more commonly performed clinically as gastrointestinal pancreatic drainage after PD. Three randomized control trials have shown PG and pancreaticojejunostomy (PJ) to be similar regarding pancreatic fistula rates[15-17] and a meta-analysis concluded that PG and PJ were not different in terms of pancreatic fistula or overall morbidity rate[18]. Nevertheless, McKay *et al*[19] using meta-analytical techniques found that the results suggested that PG rather than PJ for reconstruction of the pancreatic remnant after PD resulted in a significant decrease in pancreatic fistula or leakage. Recently, Shen *et al*[20] showed similar results in their own meta-analysis.

Many surgeons are worried about the inactivation of pancreatic enzymes and deterioration of pancreatic exocrine function due to the reflux of gastric juice into the pancreatic main duct when PG is used as the reconstruction procedure.

The aim of this article is to review the repercussion on exocrine pancreatic function according to the type of pancreatic anastomosis performed after PD. We also report our results in pancreatic exocrine function evaluated by fecal elastase test in a series of patients undergoing PD.

THEORETICAL CONSIDERATIONS OF PANCREATICODUODENECTOMY RESECTION

In a PD the possibilities regarding gastric resection are antrectomy (distal gastrectomy), or gastric-sparing techniques such as pylorus-preserving procedure, or pylorus resection with the cutting line just before the pylorus ring preserving the most part of the stomach. A greater weight loss can be expected when gastric resection is performed rather than with pylorus-preserving procedure or pylorus resection PD[2-4].

A soft, non-fibrotic gland with a small pancreatic duct increases significantly the risk of subsequent pancreatic leakage. Several techniques have been described to deal with this kind of pancreatic remnant[21]. We consider the PG anastomosis in which the pancreatic remnant is telescoped into the gastric lumen[22] as the first choice in those situations (Figures 1 and 2).

Pros and cons may be argued for each kind of anastomosis. Some groups report worse functional results with PG than PJ after PD. Their explanation for this feature is that reflux of gastric juices in the main pancreatic duct causes inactivation of the pancreatic enzymes and insufficiency of the remnant pancreas. This is a theoretical argument. In contrast, in PJ anastomosis the activation of pancreatic exocrine secretion can occur more easily in the presence of intestinal enterokinase and bile, which may irritate the remnant pancreas *via* the activation of trysinogen and chymotrypsinogen[9,23]. The activated enzymes may breakdown the anastomosis. This is also another theoretical argument without evidence-based clinical data.

Unless clinical consequences of the PG reconstruction result in greater deterioration than the potential benefits of this technique, PG is a safer anastomosis when dealing with a soft pancreatic parenchyma.

CLINICAL CONSIDERATIONS OF PANCREATIC EXOCRINE INSUFFICIENCY

Classical studies[24,25] have demonstrated that the defective digestion of protein, fat, and starch is not observed until the secretion of lipase, trypsin, and amylase is less than 10% of its normal values. The most frequently described sign of pancreatic exocrine insufficiency after resectional surgery is steatorrhoea[26], *i.e.*, stool fat content of more than 7 g/d, which may associate abdominal pain, flatus, and mostly weight loss. Fat malabsorption occurs when pancreatic lipase and trypsin decrease below 5% of normal values[27].

Halloran *et al*[28] considered that the high rate of impaired fat absorption in some series using PG, could be largely attributed to pancreatic enzyme degradation by gastric juice and acid. On the other hand, Johnson[29] studied gastric and pancreatic function after a Whipple operation with duct-to-mucosa PG in six out of 50 patients who agreed to undergo endoscopy and gastric intubation test. All patients had normal gastric secretion and all but one patient had demonstrable amylase and lipase activity in the gastric aspirate. The patient with no detectable enzyme activity had no clinical pancreatic insufficiency and had very high basal values of gastric secretion and a very high peak acid output (22 mmol/h). The explanation from the author is that although pancreatic enzymes are inactivated at low pH, the conditions found

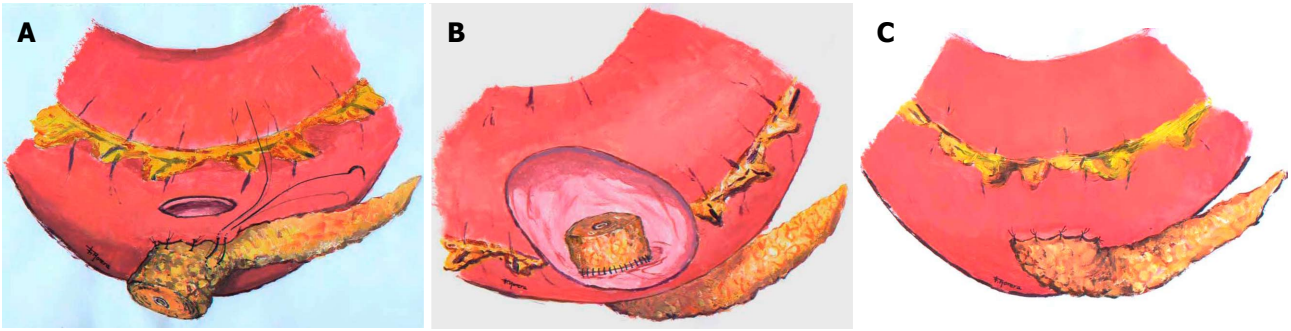

Figure 1 Pancreaticogastrostomy. A: A gastrotomy is performed in the posterior wall of the stomach, and a first layer of stitches are applied approximating gastric serosa to the pancreatic stump; B: The pancreas is telescoped into the gastric lumen, and two pancreato-mucosa running sutures complete the second layer of the anastomosis; C: The final step of the anastomosis is concluded applying the last sero-pancreatic outer stitches.

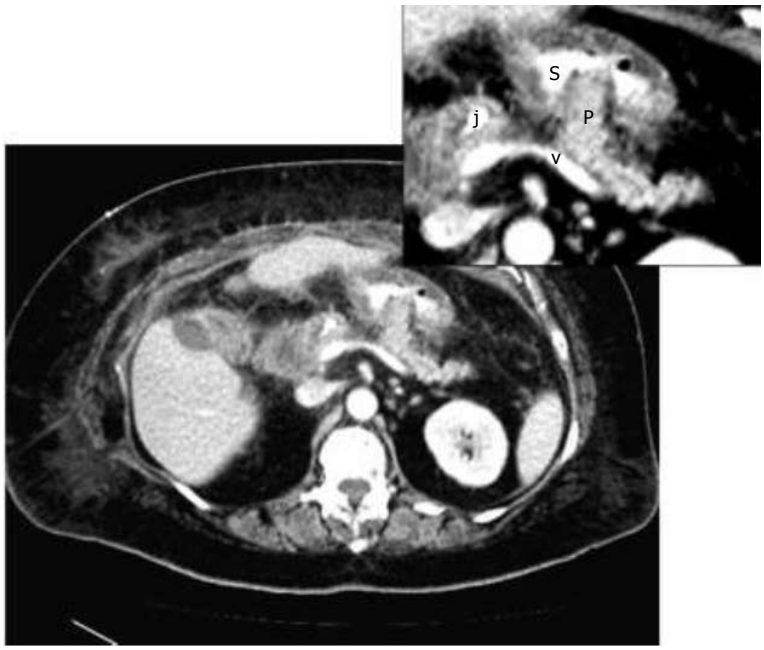

Figure 2 A scanner of the pancreaticogastrostomy in the early postoperative term. In enlarged view. p: Pancreatic stump throw the gastric wall; s: Gastric lumen containing oral contrast media; v: Splenic vein draining to the portal vein on the right side of the patient; j: High density image corresponding to the staplers of the cutting edge of the jejuna limb used in the hepatico-jejunostomy.

in the stomach immediately after a meal will be favorable for normal activity of the pancreatic enzymes. Therefore, the buffering capacity of the food may protect the pancreatic enzymes from denaturation at the time when they are required for digestion, and the PG may not be detrimental in the exocrine pancreatic function. When a PJ is performed, there is no concern about the acid pH, but the changing anatomy may provide a negative feedback following a high-caloric jejunal load which results in reduced exocrine secretion[30].

Regardless of the type of reconstruction, PD survivors should be carefully followed up for evidence of pancreatic endocrine and exocrine insufficiency[31]. When the clinical evolution of the patient demonstrates signs of pancreatic insufficiency, pancreatic enzyme replacement therapy should be routinely considered[21].

Direct pancreatic function tests have the highest sensitivity and specificity for detection of exocrine pancreatic insufficiency and are subsequently the "gold standard" for testing pancreatic function. Nevertheless, because they are cumbersome, time-consuming, and require tube placement, tubeless indirect tests of pancreatic function have been introduced. Among them, the 72-h stool fat analysis (van de Kamer test) remains the standard test to diagnose and quantify fat malabsorption[32,33]. This test requires patient compliance that is rarely obtained, and therefore its clinical use is limited.

Fecal immunoreactive elastase test is considered one of the most satisfactory pancreatic function tests for screening of pancreatic insufficiency[34]. Fecal elastase-1 measurement has been suggested to support exocrine insufficiency after pancreatic resection[19,35]. Elastase does not interfere with pancreatic enzyme supplements, and therefore the results are not affected by pancreatic enzyme replacement therapy when patients are under oral pancreatic enzyme treatment.

We have reviewed the data on fecal elastase from a series of PD performed at our centre by using both types of pancreatic anastomosis, PG and PJ. The pancreaticogastrostomy was performed in two layers, with intussusception of the pancreatic stump into the gastric lumen, an inner layer suturing the pancreatic stump to the gastric mucosa, and an outer layer approximating the pancreas to the gastric serosa. Elastase was measured by ELISA

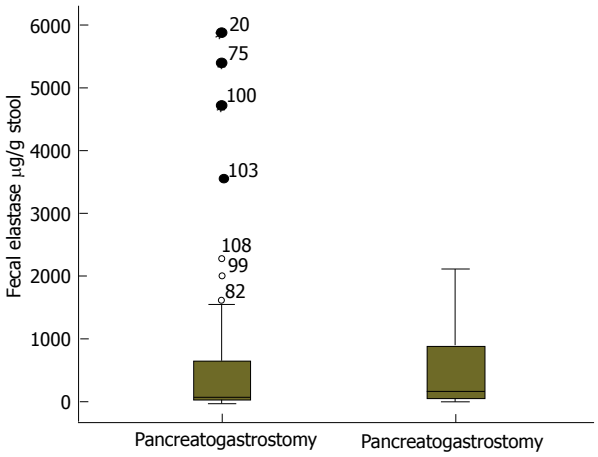

Figure 3 Fecal elastase levels in pancreatico-gastrostomy group and pancreatico-jejunostomy group. Means are depicted with horizontal bars.

to estimate the postoperative pancreatic exocrine function. Stool elastase levels were available in 108 patients, 76 PG and 32 PJ. The average age was 62.7 years ± 10.9, mostly men (64.8%). Malignancy was the most predominant pathological diagnosis (pancreatic adenocarcinoma, ampuloma, or cholangiocarcinoma) and no histology of chronic pancreatitis was found in the specimens. Fecal elastase levels are considered normal when they are above 200 μg/g stool. The mean fecal elastase after PD in our series was of 57.9 μg/g ± 104.3. The mean fecal elastase in the PG group was 61.1 μg/g ± 116.4; and it was 50.20 μg/g ± 68.5 in the PY group (Figure 3). The statistical analysis did not show significant difference between elastase levels in both groups ($P = 0.622$). There is an evident decrease in stool elastase levels of patients after PD. This decrease is not influenced by the type of pancreatic drainage used.

Non-alcoholic fatty liver disease has been described after pancreaticoduodenectomy[36-38] as a late-phase complication. This was not assessed in our patients; nevertheless this topic may be of concern in patients with long-term survival with chronic pancreatitis and it may deserve special interest in future studies.

In summary, we consider PG as the elective pancreaticointestinal drainage method after pancreaticoduodenectomy when dealing with the soft parenchyma. Pancreatic functional concerns about this kind of reconstruction do not support its rejection. Regardless the type of pancreatic anastomosis performed in the PD, the pancreatic exocrine function after pancreatic resection should be surveyed.

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TOPIC HIGHLIGHT

Jose Manuel Ramia, MD, PhD, FACS, *Series Editor*

Radiology of pancreatic neoplasms: An update

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Author contributions: Gijón de la Santa L designed research; Klein LM is a native speaker of English and revised the manuscript; Gijón de la Santa L, Pérez Retortillo JA were responsible for literature search and picture selection; Camarero Miguel A, Klein LM contributed to the literature review; all authors have contributed to the performed research and wrote the paper.

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Abstract

Diagnostic imaging is an important tool to evaluate pancreatic neoplasms. We describe the imaging features of pancreatic malignancies and their benign mimics. Accurate detection and staging are essential for ensuring appropriate selection of patients who will benefit from surgery and for preventing unnecessary surgeries in patients with unresectable disease. Ultrasound, multidetector computed tomography with multiplanar reconstruction and magnetic resonance imaging can help to do a correct diagnosis. Radiologists should be aware of the wide variety of anatomic variants and pathologic conditions that may mimic pancreatic neoplasms. The knowledge of the most important characteristic key findings may facilitate the right diagnosis.

Key words: Pancreas; cancer; Radiology; Computed tomography; Magnetic resonance imaging; Surgery; Pancreatic neoplasms

Core tip: Diagnostic imaging is an important tool to evaluate pancreatic neoplasms. We describe and illustrate the imaging features and key findings of pancreatic malignancies and their mimics. The knowledge of radiologic findings is relevant to do an accurate diagnosis that allows a proper management and should be known not only for radiologists but by physicians that comprise multidisciplinary teams.

Gijón de la Santa L, Pérez Retortillo JA, Camarero Miguel A, Klein LM. Radiology of pancreatic neoplasms: An update. *World J Gastrointest Oncol* 2014; 6(9): 330-343 Available from: URL: http://www.wjgnet.com/1948-5204/full/v6/i9/330.htm DOI: http://dx.doi.org/10.4251/wjgo.v6.i9.330

INTRODUCTION

Diagnostic imaging is an important tool to evaluate pancreatic neoplasms. Accurate detection and staging are essential for ensuring appropriate selection of patients who will benefit from surgery and for preventing unnecessary surgeries in patients with unresectable disease[1,2]. Ultrasound (US), multidetector computed tomography (MDCT) with multiplanar reconstruction and magnetic resonance imaging (MRI) can help to do a correct diagnosis[3,4].

A wide variety of anatomic variants and pathologic conditions exist that may mimic pancreatic neoplasms. Pancreas such as pancreas divisum or anular pancreas may cause enlargement of the pancreatic head and be mistaken for a tumoral mass. Non-distended adjacent bowel, gastric fundus, duodenal diverticula, duplications[2,5-7] accessory spleen or splenosis may also mimic a pancreatic mass[8]. Chronic pancreatitis may be indistinguishable from neoplasm on the basis of morphologic at MRI and MDCT[9] (Figure 1). Positron emission tomography (PET) with 2-[18F]-fluoro2-deoxy-d-glucose (FDG)/MRI fusion image significantly improved accu-

Table 1 Pancreatic tumors

Pancreatic tumors	
Primary (95%)	
Solid tumors	
	Pancreatic adenocarcinoma (85%-95%)
	Pancreatic neuroendocrine tumor
	Solid pseudopapillary tumor
	Pancreatoblastoma
	Pancreatic lymphoma
Cystic tumors	
	Serous cystadenoma
	Mucinous cystic neoplasm
	Intraductal papillary mucinous tumor of the pancreas
Metastatic lesions (5%)	

racy compared with that of PET/CT (in differentiating pancreatic cancer from benign lesions 96.6% *vs* 86.6%)[10].

Enlarged peripancreatic nodal chains and disease in surrounding structures can mimic pancreatic masses (gastric fundus neoplasm, small bowel tumors, renal or adrenal masses, *etc*.). The existence of fat planes between the nodes or tumoral masses and the pancreatic gland or displacement of the pancreas may be useful to distinguish these lesions from a pancreatic mass[6] (Figure 2). Choledochal cysts may simulate a cystic mass in the head of the pancreas[11].

True pancreatic masses can be classified in primary or metastatic lesions (Table 1).

PRIMARY PANCREATIC LESIONS

Primary pancreatic masses will be classified on the basis of its radiologic appearance in solid or cystic lesions.

SOLID LESIONS OF THE PANCREAS

Pancreatic adenocarcinoma

Pancreatic adenocarcinoma accounts for 85%-95% of all pancreatic malignancies and is the fourth leading cause of cancer-related deaths. Most patients are 60-80 years of age, and males are affected twice as often as females[3,4]. Of these tumors, 60%-70% are located in the pancreatic head, 10%-20% in the body, and 5%-10% in the tail. Diffuse glandular involvement occurs in 5% of cases[2,3]. Surgery is the only cure, with a postoperative 5-year survival rate of 20%[3,4]. Unresectable disease is seen at presentation in 75% of patients (Figure 3).

Dual-phase (arterial and portal) contrast material–enhanced MDCT is the established technique for evaluating pancreatic adenocarcinoma. Arterial phase imaging (performed 20-40 s after contrast agent injection) allows optimal visualization of the tumor and peripancreatic arteries (Figure 4). Portal phase imaging (performed 50-70 s after injection) is optimal for assessing the peripancreatic veins and detecting metastatic disease to the liver[3] (Figure 5). After intravenous contrast administration most tumors are hypoattenuating (Figure 6).

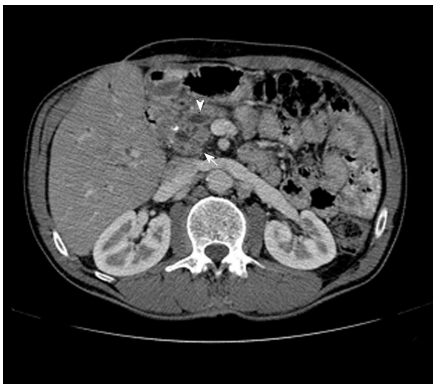

Figure 1 Multidetector computed tomography image. Multidetector computed tomography shows enlargement of the pancreatic head (arrow), with dilatation and beading of the pancreatic duct (arrowhead) and dilatation of the extra- and intrahepatic bile ducts. A focal calcification can also be visualized. These findings matched with the definite diagnosis of a chronic pancreatitis.

No pancreatic mass is visualized in 10% of cases, since the tumor may be isoattenuating. The presence and location of a mass may be inferred from secondary signs such as mass effect, an abnormal convex contour of the pancreas, ductal obstruction, and vascular invasion[2-4] (Figures 7 and 8). Tumors in the pancreatic head may cause dilatation of both common bile duct and the main pancreatic duct (MPD), known as the "double duct sign"; whereas tumors in the pancreatic body may cause upstream MPD dilatation (Figure 9A). A circumferential soft-tissue cuff around the peripancreatic vessels with loss of the perivascular fat plane denotes vascular invasion. A sensitivity of 84% and a specificity of 98% for invasion are reported if the tumor is contiguous with more than 50% of the vessel circumference[1] (Figure 9B). Other features suggesting vascular invasion include vessel deformity, thrombosis, and development of collateral vessels[12]. Cystic-necrotic degeneration, an uncommon feature of adenocarcinoma, is present in 8% of cases[13,14]. Metastases are most commonly found in the liver (Figure 5B) and peritoneum (Figure 9C)[2,3].

Adenocarcinoma has low signal intensity on T1 and T2 weighted MRI secondary to its scirrhous fibrotic nature (Figure 10). As at MDCT, the hypovascular tumor enhances less than the normal pancreas at MRI (Figure 11). MRI has better contrast resolution than MDCT and is superior in detecting small tumors and metastases[15]. Diffusion-weighted (DW) MRI allows the assessment of thermally induced random molecular motion in biologic tissues and generates representative apparent diffusion coefficient (ADC) values[16-18]. The use of DW MRI may allow earlier detection of pancreatic tumours, since these neoplasms have increased signal intensity on diffusion-weighted images and relatively low ADC values because of the restricted diffusion associated with fibrosis (Figure 12). In addition, DW MRI may be helpful in the detection of metastases in the liver and lymph nodes[16,17].

Endoscopic US has a recognized role in the detection and staging of small tumors. It can help detect masses as small as 0.2 cm. Endoscopic US can clarify equivocal find-

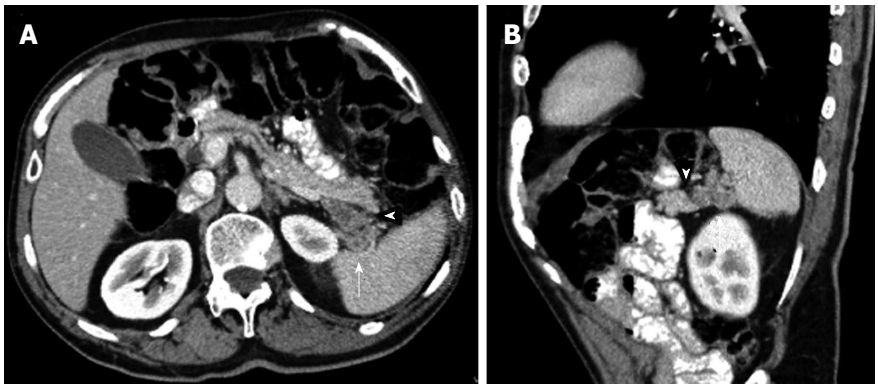

Figure 2 Axial contrast enhanced multidetector computed tomography image. A: Depicts a nodular peripancreatic mass localized between the pancreatic tail (arrowhead) and the splenic hilum (arrow), each well separated by fat planes; B: The sagittal reformatted contrast enhanced multidetector computed tomography image allows a better identification of the surrounding fat planes (arrow and arrowhead) enabling the exclusion of a pancreatic dependency. This mass actually turned out to be a tumoral implant of a gastric neoplasm.

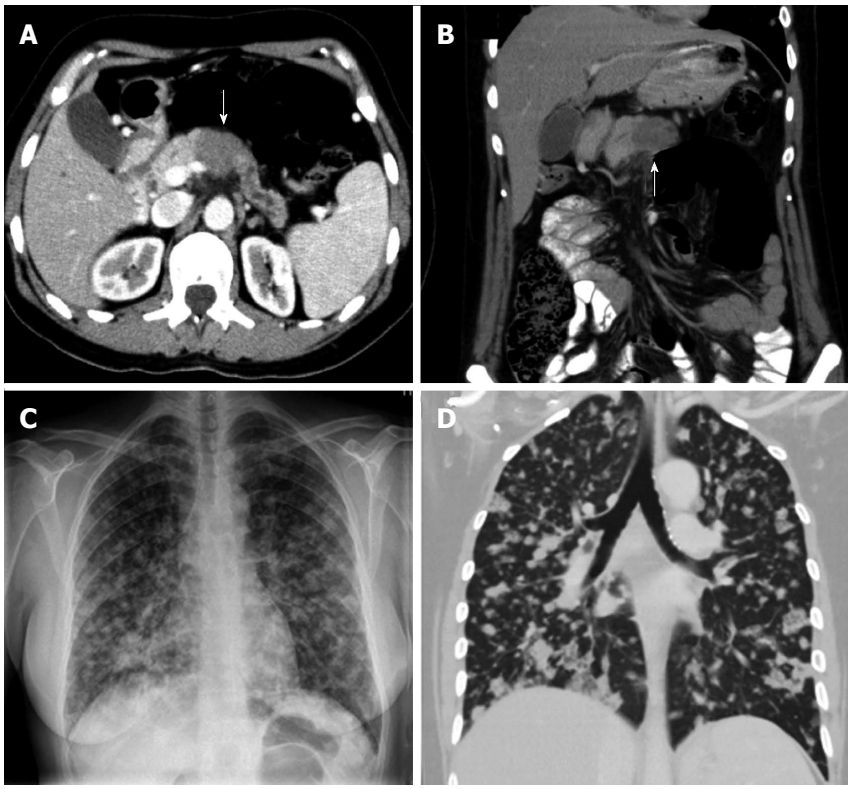

Figure 3 Unresectablility of a pancreatic adenocarcinoma. Contrast enhanced multidetector computed tomography (MDCT) image (A) and coronal reformation image (B) shows dilatation of the distal pancreatic duct caused by a hypodense tumor (arrow) in the pancreatic body. On plain film (C) and coronal reformation image on MDCT (D) of the same patient multiple lung metastases of his pancreatic carcinoma are evident - a definite criteria for unresectability.

ings at MDCT or MRI and allows biopsy of suspect lesions. Adenocarcinoma appears as an ill-defined, heterogeneous hypoechoic mass at endoscopic US[3] (Figure 13).

PET is an emerging technique for characterizing tissue on the basis of functional rather than morphologic information. The principle of FDG PET is that malignant tissues have greater uptake and retention of FDG than does normal tissue due to enhanced glucose metabolism. Pancreatic adenocarcinoma generally shows intense focal FDG uptake. The biggest potential impact of FDG PET is in the detection of small metastases, an area in which MDCT and MRI generally underestimate lesions[3].

Pancreatic neuroendocrine tumor

Pancreatic neuroendocrine tumors (NETs) account for 1%-5% of all pancreatic tumors and typically manifest in patients aged 51-57 years. Most cases are sporadic, but association with syndromes such as multiple endocrine neoplasia type 1, von Hippel-Lindau syndrome, neurofibromatosis type 1, and tuberous sclerosis has been observed. Tumors tend to be multiple when associated with syndromes.

NETs are classified into functioning and nonfunc-

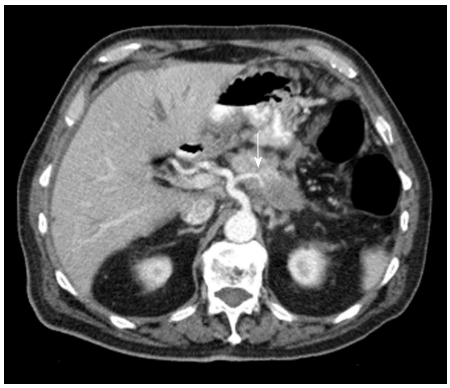

Figure 4 Axial contrast enhanced multidetector computed tomography image. Arterial phase imaging allows optimal visualization of the pancreatic neoplasm and peripancreatic arteries: the shown hypodense mass compromises the splenic artery (arrow). Pancreatic adenocarcinoma was proven by biopsy.

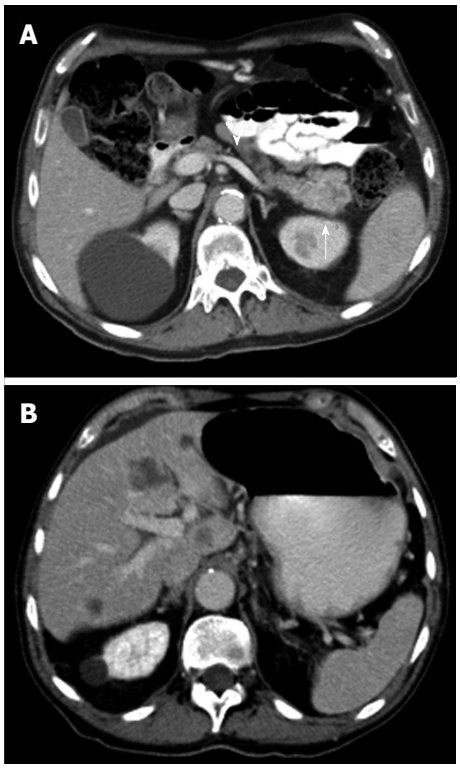

Figure 5 Contrast enhanced multidetector computed tomography image. A: In portal venous phase depicts a mass (arrow) in the pancreatic tail with permeability of the splenic vein (arrowhead); B: Focal round focal hypodensities with different sizes, localized in both hepatic lobules, represent metastatic spread to the liver. Pancreatic adenocarcinoma was proven by biopsy.

tioning tumors. Functioning tumors produce symptoms related to excessive hormone production. In general, functioning tumors manifest early in the course of disease. Nonfunctioning tumors manifest when they are large, due to mass effect. Risk of malignancy increases with tumor size (especially in tumors > 5 cm). Because of this fact 90% of nonfunctioning tumors are malignant at presentation[19].

Small tumors are generally solid and homogeneous, whereas larger tumors are heterogeneous and may show

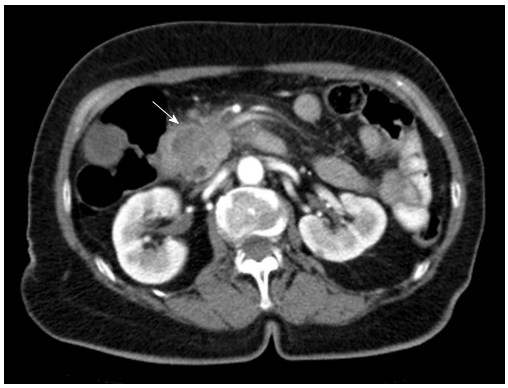

Figure 6 Axial multidetector computed tomography image. Pancreatic tumor, localized in the pancreatic head (arrow), is hypodense in relation to the pancreatic parenchyma after contrast administration.

variable amounts of cystic-necrotic degeneration and calcification[3,19,20] (Figure 14).

NETs have a rich vascular supply and therefore enhance avidly during the arterial phase, enhancing more rapidly and intensely than the normal pancreas. That finding helps differentiate NETs from the more common adenocarcinoma which is hypovascular. Homogeneous enhancement is typical for small tumors (less than 2 cm), whereas larger lesions tend to show heterogeneous enhancement.

When NETs have a predominantly cystic component MDCT and MRI show a hypervascular enhancement in the nonnecrotic or nondegenerated portions of the tumor. Cystic areas are typically hyperintense at MRI on T2-weighted images (Figure 15).

Metastases to lymph nodes and solid organs such as the liver may have an enhancement pattern similar to that of the primary tumor (Figure 16). Cystic metastases to the liver may also be seen[3,19].

Solid pseudopapillary tumor

Solid pseudopapillary tumor (SPT) accounts for 1%-2% of all pancreatic tumors. It is most common in young females (mean age, 25 years)[21]. SPT has a low malignant potential with an excellent prognosis following complete resection.

SPT is typically a large (mean, 9 cm), slow-growing, well-encapsulated mass[21,22]. It most commonly occurs in the pancreatic tail. SPT has a tendency to displace rather than invade surrounding structures and rarely causes obstruction of the bile duct or pancreatic duct. MDCT usually demonstrates a well-encapsulated lesion with varying solid and cystic components owing to hemorrhagic degeneration[23]. Hemorrhage may progress to cystic changes within the lesions in approximately 20% of cases. Degenerated areas may mimic certain features of larger NETs. However, the peripheral portions of solid and papillary epithelial neoplasms do not demonstrate the hypervascularity typical of NETs[21]. SPT shows peripheral heterogeneous enhancement with central cystic spaces[24,25].

MRI typically demonstrates a well-defined lesion with

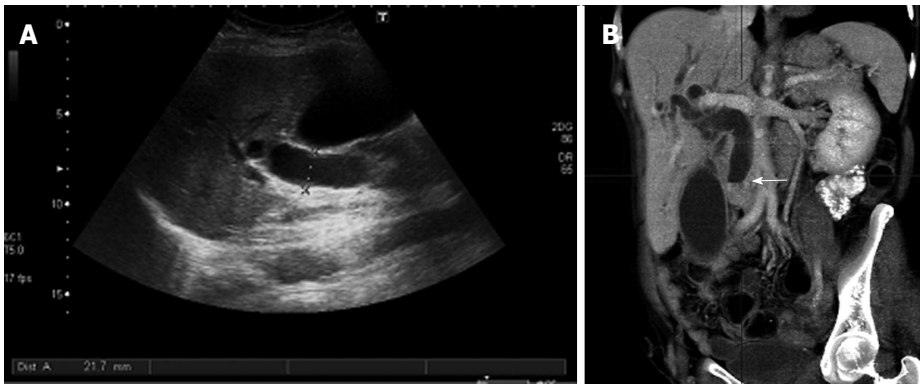

Figure 7 Indirect signs of pancreatic neoplasms. Transverse ultrasound image (A) shows a markedly dilated common bile duct, also seen on the coronal reformation image of multidetector computed tomography (B) where the dilated duct terminates abruptly at the level of the pancreatic head (arrow).

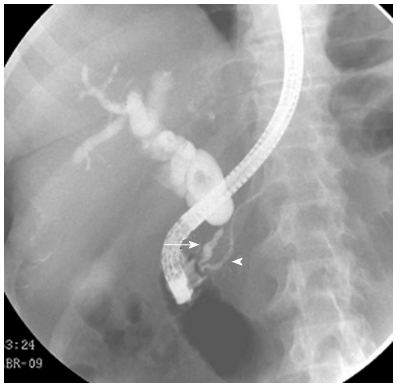

Figure 8 Endoscopic retrograde cholangiopancreatography. A short segment of narrowing causing stenosis of the common bile duct was recognized (arrow), without affection of the main pancreatic duct (arrowhead). Pancreatic adenocarcinoma was proven by biopsy.

heterogeneous signal intensity on T1- and T2-weighted images. Peripheral calcification is present in 30% of cases[21]. The pseudocapsule (composed of compressed pancreatic tissue and reactive fibrosis) has low attenuation at MDCT and low signal intensity at T_1- and T_2-weighted MRI.

Internal hemorrhagic and cystic degeneration is the hallmark of SPT due to the fragile vascular network of the tumor[3,26]. Although most SPTs exhibit benign behavior, malignant degeneration does occur. Metastases are uncommon, occurring in 7%-9% of cases, mostly to the liver, omentum, and peritoneum[27].

Pancreatoblastoma

Pancreatoblastoma accounts for 0.2% of all pancreatic tumors and is the most common pancreatic tumor in young children (mean 5 years)[3,28]. Pancreatoblastoma rarely occurs in adults; when it does, however, the tumor is generally more aggressive. The serum alpha-fetoprotein level is elevated in 25%-33% of cases[29].

Pancreatoblastoma is typically slow growing and generally manifests as an asymptomatic large mass (mean, 10 cm). Because of the large size of the mass at presentation, in 50% of cases it is not possible to identify the

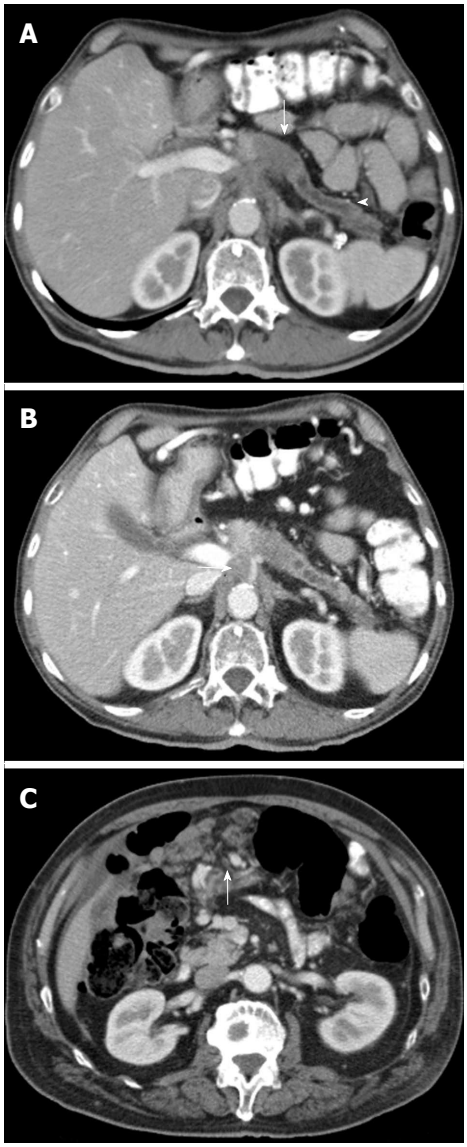

Figure 9 Axial contrast enhanced multidetector computed tomography image A: Focal hypodense mass in the body of the pancreas (arrow), with upstream dilatation of the main pancreatic duct (arrowhead). Pacreatic adenocarcinoma was histologically proven; B: Image depicts a circumferential soft tissue cuff around the celiac trunk according to vascular invasion (arrow); C: Image shows multiple peritoneal metastases in a patient with a pancreatic tumor (arrow).

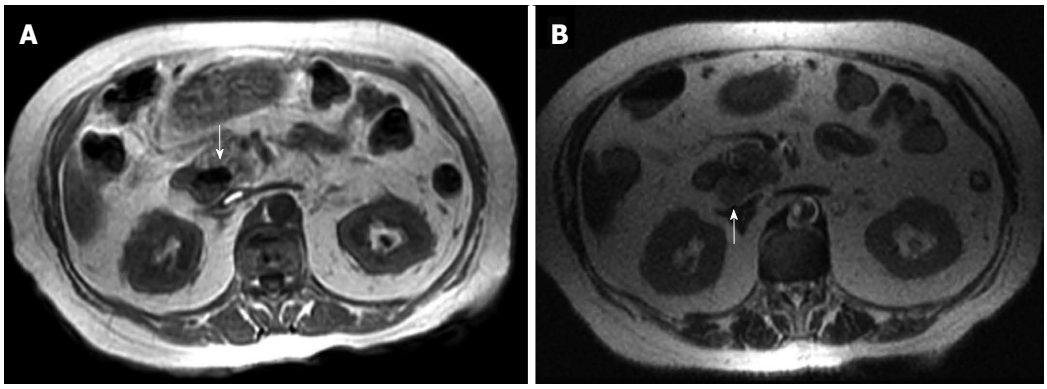

Figure 10 Adenocarcinoma has low signal intensity on T1 (A) and T2 (B) weighted magnetic resonance imaging (arrows).

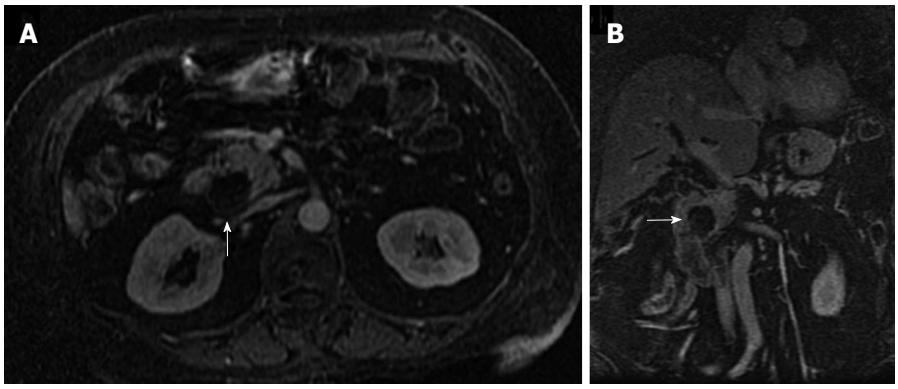

Figure 11 Axial arterial-phase gadolinium-enhanced T1-weighted fat-suppressed gradient-recalled echo magnetic resonance imaging (A) and coronal reformatted (B) show no enhancement of the hypovascular tumor in the pancreatic head (arrow). Pancreatic adenocarcinoma was proven by biopsy.

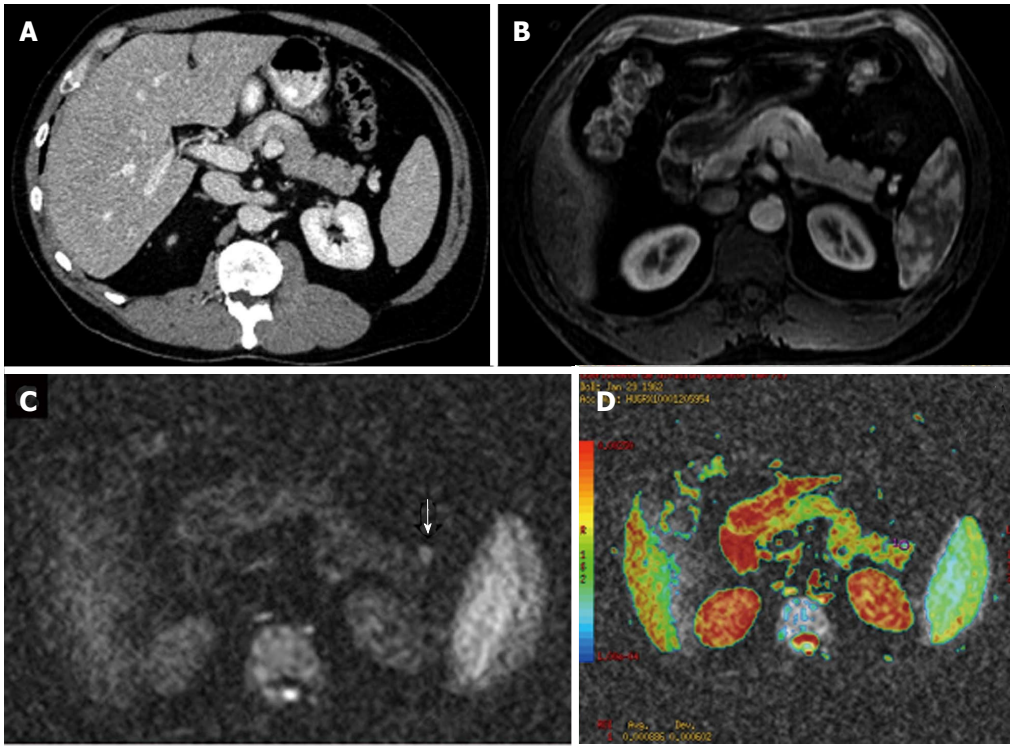

Figure 12 Use of diffusion-weighted magnetic resonance imaging in the earlier detection of pancreatic tumours. Axial contrast enhanced multidetector computed tomography image (A) and axial arterial-phase gadolinium-enhanced T1-weighted fat-suppressed gradient-recalled echo magnetic resonance image (B) do not depict any abnormality. Axial diffusion-weighted magnetic resonance imaging (C) demonstrates a focal increased signal intensity (arrow) and low apparent diffusion coefficient values in the color coded images (D).

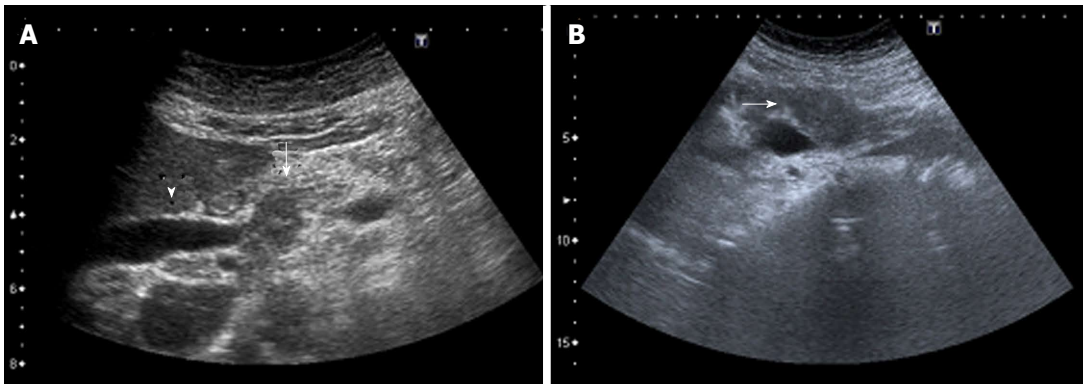

Figure 13 Ultrasound images (A, B) of an ill-defined, heterogeneous hypoechoic mass (arrow) in the pancreas obstructing the common bile duct (arrowhead).

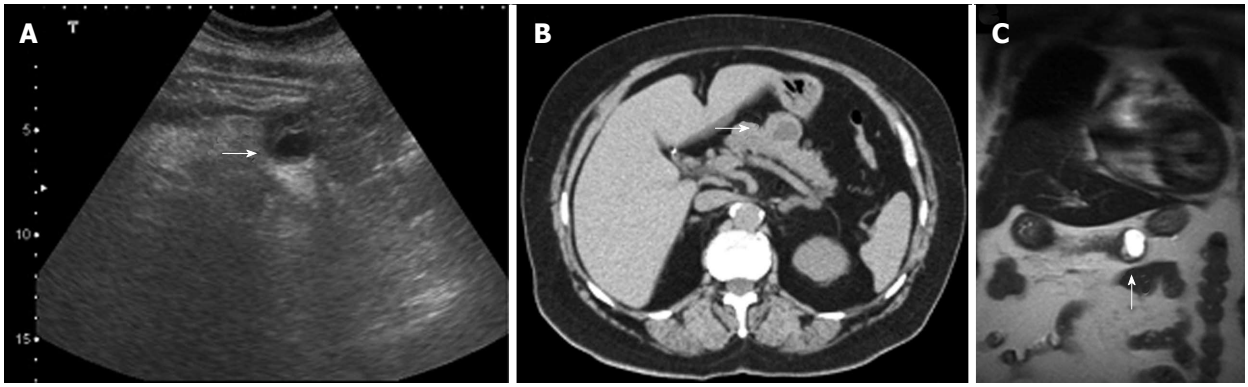

Figure 14 Pancreatic neuroendocrine tumor. Ultrasound images (A), axial unenhanced multidetector computed tomography and coronal magnetic resonance T2-weighted image show a round, heterogeneous mass, localized in the pancreatic body, with variable amounts of cystic-necrotic degeneration (arrows).

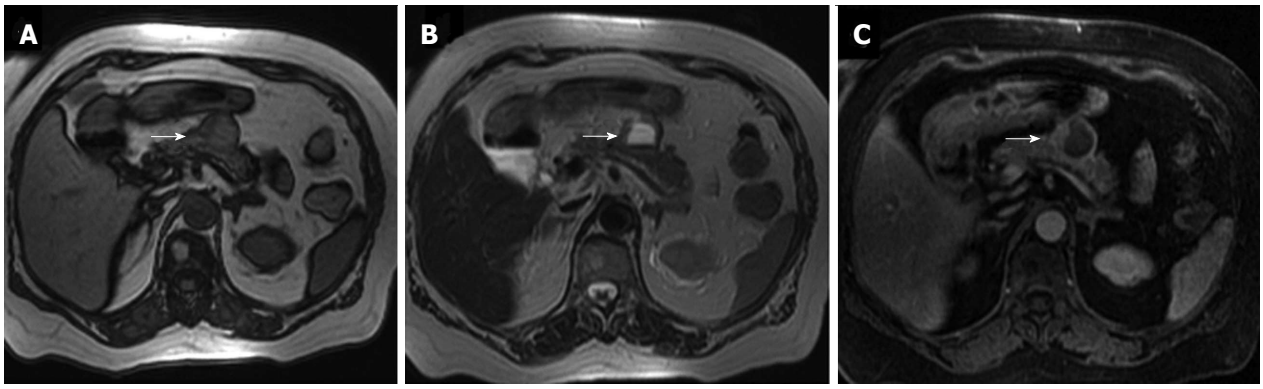

Figure 15 Same patient shown in figure 14. Magnetic resonance axial gradient T1 out-of-phase image (A) and T1 fat-suppressed sequence (C) show a hypointense signal in the liquid component of the lesion whereas it reveals a hyperintense signal in the T2-weighted sequence (B) (arrows).

organ of origin at radiology[30]. Therefore, differentiation from other pediatric tumors arising from adjacent organs (*e.g.*, neuroblastoma, Wilms tumor, hepatoblastoma) is challenging, and biopsy is generally required to establish the diagnosis. Metastases occur mostly to the liver.

At US, the mass is heterogeneous with hypoechoic cystic spaces and hyperechoic internal septa[28]. At MDCT, pancreatoblastoma generally manifests as a multiloculated inhomogeneous mass with enhancing septa[28]. On MRI the tumor has low to intermediate signal intensity on T_1- and high signal intensity on T_2-weighted images, and shows mild contrast enhancement.

Pancreatic lymphoma

Pancreatic lymphoma is most commonly a B-cell subtype of non-Hodgkin lymphoma. Secondary lymphoma is the dominant form and is the result of direct extension from peripancreatic lymphadenopathy. Primary pancreatic lymphoma is rare, representing 0.5% of pancreatic tumors. It is more common in immunocompromised patients[31].

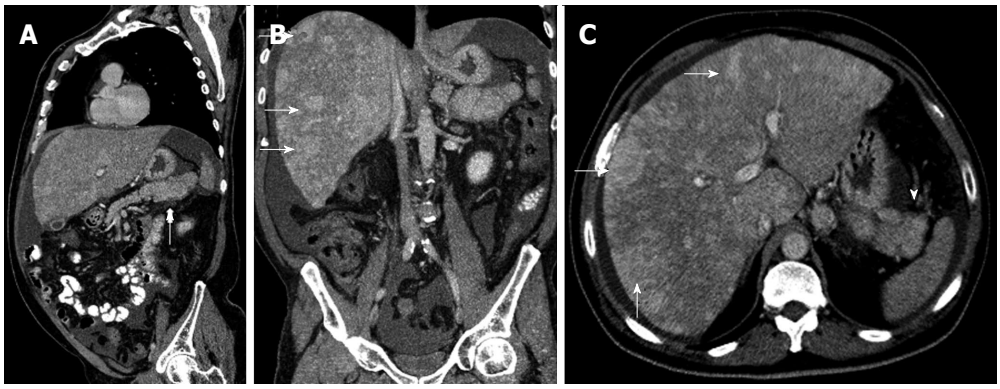

Figure 16 Sagittal multidetector computed tomography image. A: A heterogeneous pancreatic mass (arrow); B, C: Coronal (B) and axial (C) multidetector computed tomography images show multiple hypervascular metastases in the liver (arrows), showing the same enhancement pattern of the primary mass. Neuroendocrine pancreatic tumor and metastases were histologically proven.

Two morphologic patterns of pancreatic lymphoma are recognized: a focal well-circumscribed form and a diffuse form. The focal form occurs in the pancreatic head in 80% of cases and has a mean size of 8 cm. It typically has uniform low attenuation at MDCT. At MRI, it has low signal intensity on T_1- and intermediate signal intensity on T_2- weighted images and shows faint contrast enhancement. The diffuse form is infiltrative leading to glandular enlargement and poor definition, features that can simulate the appearance of acute pancreatitis[32,33].

CYSTIC LESIONS OF THE PANCREAS

Cystic lesions accounts for 10%-15% of all pancreatic neoplasms and represents < 5% of all malignant pancreatic tumors.

Unilocular cysts are well defined lesions without internal septa, calcification or internal soft-tissues nodules. When small (< 3 cm), these lesions are almost always benign. It is suggested to do serial imaging at 6-mo intervals for the first year and annual follow-up for a period of three years. If the cyst remains stable and the patient asymptomatic no further workup in needed[34].

Pseudocyst (encapsulated fluid collections without necrosis after 4 wk from onset of acute pancreatitis) is the most common unilocular cyst[34,35]. It is important to ask for the patient's history because a cystic lesion in a patient with a clinical history of pancreatitis is almost always a pseudocyst.

Imaging studies shows a rounded cystic mass with a thick wall. After intravenous contrast administration mild wall enhancement is demonstrated (Figure 17). If we detect a solid intracystic component, the lesion is not a pseudocyst. Other image findings that support this diagnosis are inflammation, atrophy or pancreatic calcifications. Cystic neoplasm may appear as uni or multilocular masses.

Serous cystadenoma

It is a benign lesion which typically occurs in older women. The cystic components range from millimeters to 2 cm. When the lesion grows a central scar and coarse calcification may be seen (30%). This calcified scar is highly specific and virtually pathognomonic[36] and is best demostrated at CT.

MRI shows a cluster of small cyst without visible communication within the cyst or the pancreatic duct. These cysts are hyperintense on T_2-weighted images. Central calcified scar is seen as a signal void at MRI (Figure 18). Enhancement of fibrous septa between the cysts are seen on delayed images.

Mucinous cystic neoplasm (mucinous cystadenoma/ cystadenocarcinoma)

This lesion has female predominance (80%) in their sixth decade of life[37]. Mucinous cystadenoma preferentially involves the body and pancreatic tail and do not communicate with the pancreatic duct.

Cross-sectional imaging is ineffective for differentiating between mucinous cystic neoplasms with and without malignant epithelium, except in cases with invasion of adjacent organs, vascular invasion, or metastatic disease. The presence of intracystic enhancing soft tissues are suspicious for malignancy. Peripheral eggshell calcifications are not frequent (16%) but such finding is specific and has a highly predictive value for malignancy.

On US mucinous cystic neoplasms appear as hypoechogenic multilocular or, less commonly, unilocular masses with posterior acoustic enhancement. Internal septations are usually visualized and better demonstrated at US than at CT[36-40].

CT shows a round to slightly lobulated mass that is well encapsulated with smooth external margins. Because the cyst contents can vary in attenuation according to the degree of hemorrhage or protein in the mucoid cysts, different levels of attenuation may be seen within the cyst cavities[37,39,41-44] (Figure 19). After intravenous contrast administration septa and peripheral wall enhancement are detected.

At MR the lesion is hypointense on T_1- and hyperintense on T_2-weighted images. This lesion may be hyperintense on T_1-weighted images due to mucinous content.

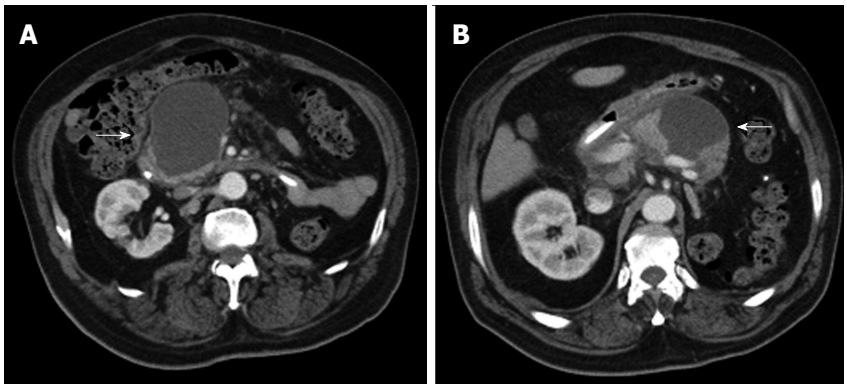

Figure 17 Axial contrast enhanced multidetector computed tomography images (A, B) reveal a homogeneously hypodense intraparenchymal fluid collection of the pancreas without any non-liquefied material in it, encapsulated completely by a thin slightly hyperdense layer (arrows). These findings are compatible with a pseudocyst in a patient with a clinical history of pancreatitis.

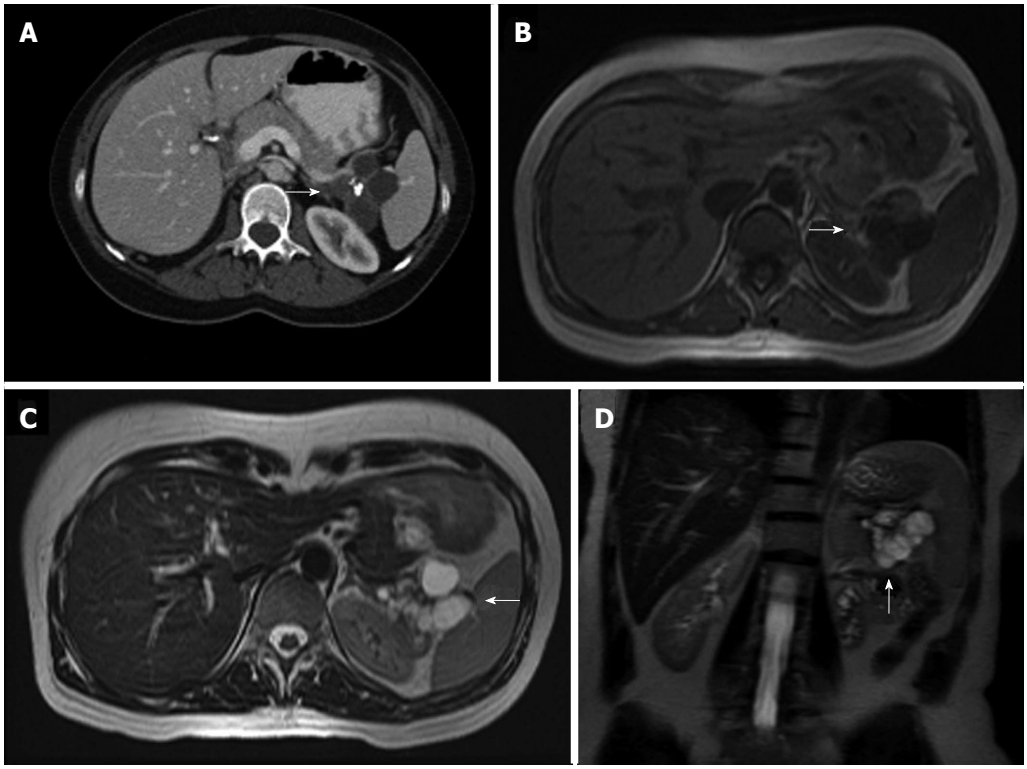

Figure 18 Axial nonenhanced multidetector computed tomography image. A: A polylobulated cystic lesion with a coarse calcification in its center (arrow), which is the phathognomonic central scar for serous cystadenoma; B-D: Magnetic resonance imaging show a cluster of small cysts (arrows), which are hypointense in T1-weighted images (B) and hyperintense in T2-weighted images (C, D), without visible communication within the cyst or the pancreatic duct. A central signal void is also identifiable.

Intraductal papillary mucinous tumor of the pancreas

Intraductal papillary mucinous tumor of the pancreas (IPMN) are most frequent indentified in elderly men. The most important features are the presence of mucin-producing tumor and cystic dilation of the main pancreatic duct, its branches or both[45,46]. The dilated ducts often contain profuse mucin. In the past, many IPMTs may have been misdiagnosed as chronic pancreatitis because of their generally benign behavior.

IPMNs may be classified as benign or malignant on the basis of the degree of dysplasia[47-50].

Preoperative determination of the presence or absence of associated invasive carcinoma is crucial; when invasive carcinoma is present, the surgical procedure may be modified to include resection of regional lymph nodes.

Main duct IPMNs are more likely to be malignant. IPMNs are frequently multifocal, and 5%-10% involve the entire pancreas.

When CT reveals a pancreatic solid mass in patients with IPMN, the lesion is probably invasive carcinoma. Other imaging features suggestive of invasive carcinoma in IPMN are the large size of the mass (> 3.5 cm), presence of mural nodules, dilatation of the main pancreatic

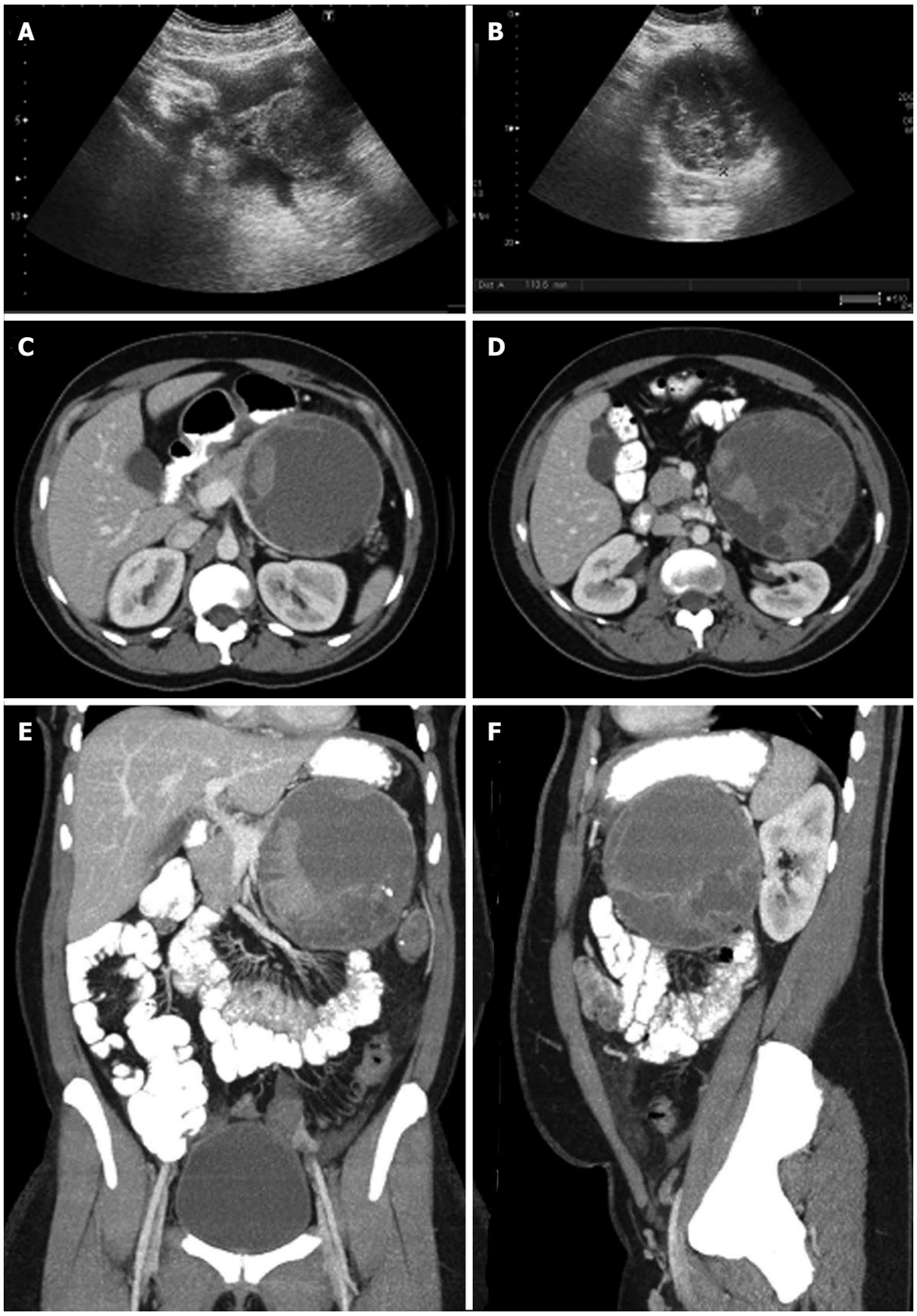

Figure 19 Ultrasound and multidetector computed tomography images. On ultrasound (A, B) a hypoechogenic multilocular mass with well-definable internal spetations and posterior acoustic enhancement can be seen. Contrast-enhanced multidetector computed tomography images (C-F) show a big round to slightly lobulated mass with an enhancing capsule and different levels of attenuation within the cyst cavities are seen. Some enhancing components are also detectable.

duct > 15 mm and multifocal involvement[49,51].

MRI is better than CT for evaluating ductal communication[52,53]. Dilatation of main pancreatic duct or multiple side branches on T_2-weighted images is the most common imaging finding[54]. Demonstrating ductal communication can be useful to differentiate between IPMNs and mucinous cystadenoma (the latter has no communication with the pancreatic ductal system) (Figure 20).

Three-dimensional contrast-enhanced ultrasonography showed similar results as compared with MRI in evaluating "IPMNs" smaller than 1 cm of diameter or greater than 2 cm[55].

METASTASES TO THE PANCREAS

Pancreatic metastases account for 2%-5% of all malignant neoplasms. Metastases are most frequently from renal cell carcinoma and lung carcinoma[56]. The progno-

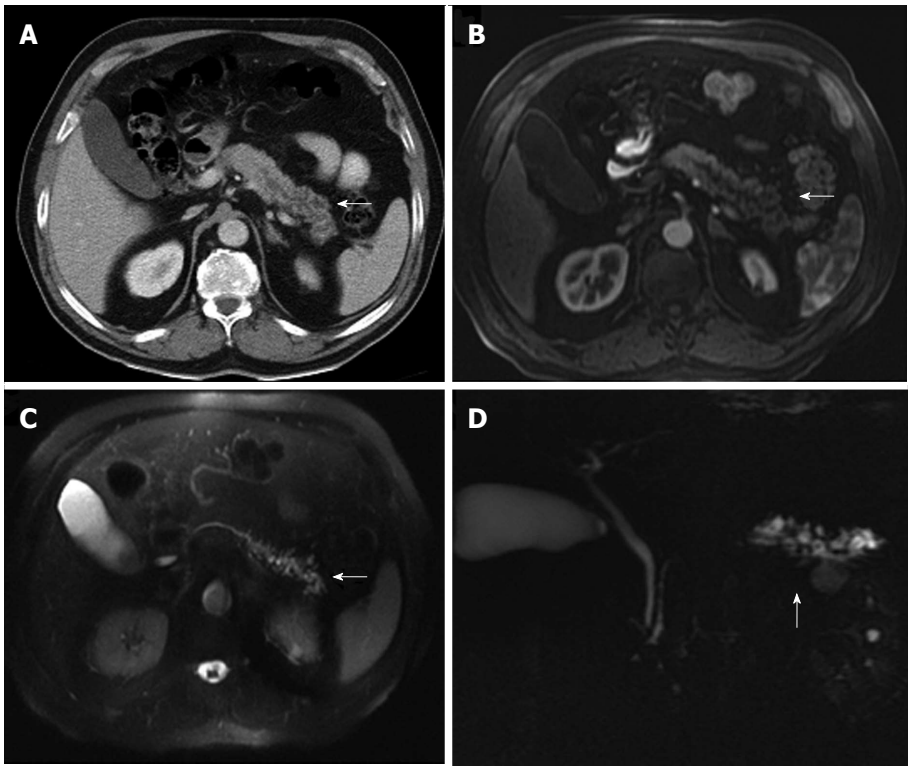

Figure 20 Multidetector computed tomography image. A: Cystic dilatation of the main pancreatic duct and some of its branches in the pancreatic tail. Ductal communication with the tumor cannot be clearly identified; B-D: In contrast-enhanced axial T1 (B) and T2-weighted (C) magnetic resonance images and in magnetic resonance imaging cholangiography (D) ductal communication can be easily detectable.

sis is generally more favorable than that for pancreatic adenocarcinoma[3] (Figure 21).

Three morphologic patterns of involvement are recognized: solitary (50%-70%), multifocal and diffuse[56,57]. At contrast-enhanced CT and MR imaging, the appearances of pancreatic metastases closely resemble that of primary carcinoma but pancreatic adenocarcinoma generally manifests as a hypoenhancing mass, whereas metastases show either peripheral enhancement (in lesions > 1.5 cm) or, less commonly, homogeneous enhancement (smaller lesions)[56,58,59].

Cystic metastases to the pancreas cannot be differentiated from mucinous cystic neoplasms radiographically. Ovarian carcinoma metastases are the most likely to manifest as a predominantly cystic mass.

A known history of primary malignant disease, combined with the presence of other metastatic foci, are helpful clues in making the diagnosis.

INTRAOPERATIVE ULTRA-SONOGRAPHY OF THE PANCREAS

Up to 40% of patients with pancreatic adenocarcinoma judged resectable at CT are found to have unresectable lesions at surgery[60,61]. Laparoscopy intraoperative US may be useful before open surgical resection to decrease the number of patients who undergo needless open surgery for resection of a tumor that ultimately proves unresectable[62]. Pancreatic adenocarcinoma appears at intraoperative US as a hypoechoic mass with ill-defined margins[60].

EVALUATION OF THE POSTOPERATIVE PANCREAS

The most common complications of the Whipple procedure are delayed gastric emptying, pancreatic fistulas, wound infection, abdominal abscess, intraabdominal bleeding, and anastomotic leakage. A pancreaticojejunal fistula is diagnosed clinically on the basis of the detection of amylase-rich fluid in the drainage. Anastomotic leaks usually occur at the pancreaticojejunal anastomosis during first 2 wk after pancreatoduodenectomy and these leaks can be diagnosed on the basis of the presence of oral contrast material in the peritoneal cavity and are associated with peripancreatic fluid collections[63,64].

Locally recurrent disease is sometimes difficult to depict on the earliest postoperative images. Locally recurrent disease appears as an infiltrating mass with soft-tissue attenuation, perineural invasion and encasement of the mesenteric vessels[65]. Perivascular cuffing in the mesenteric fat is likely inflammatory in patients with negative surgical margins and should not be mistaken for local recurrence[63].

CONCLUSION

The knowledge of some of the most important characteristic key findings of pancreatic tumors may facilitate radiologists, and especially radiographers in training,

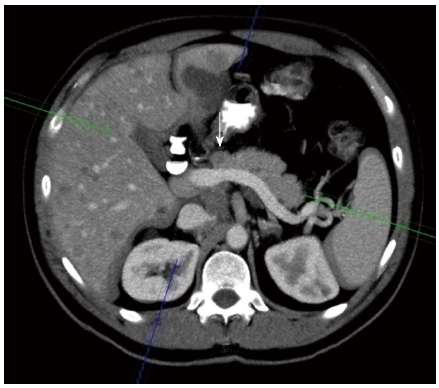

Figure 21 Oblique reformatted enhanced multidetector computed tomography image reveals a well-defined round mass in the pancreas, slightly hypodense to the pancreatic parenchyma. Pancreatic metastases from melanoma was proven. Note the liver concomitant metastases.

to do an accurate detection and staging of pancreatic neoplams in order to ensure an appropriate selection of patients who will benefit from surgery and prevent unnecessary surgeries in patients with unresectable disease.

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TOPIC HIGHLIGHT

Jose Manuel Ramia, MD, PhD, FACS, *Series Editor*

Tricks and tips in pancreatoduodenectomy

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Abstract

Pancreaticoduodenectomy (PD) is the standard surgical treatment for tumors of the pancreatic head, proximal bile duct, duodenum and ampulla, and represents the only hope of cure in cases of malignancy. Since its initial description in 1935 by Whipple *et al*, this complex surgical technique has evolved and undergone several modifications. We review three key issues in PD: (1) the initial approach to the superior mesenteric artery, known as the artery-first approach; (2) arterial complications caused by anatomic variants of the hepatic artery or celiac artery stenosis; and (3) the extent of lymphadenectomy.

Key words: Pancreas; Pancreaticoduodenectomy; Artery-first; Surgery; Lymphadenectomy; Celiac axis; Hepatic artery

Core tip: The "artery-first approach" prioritized the dissection of the origin of the superior mesenteric artery (SMA), allowing complete lymphadenectomy, safe dissection of the SMA, and accurate identification of the most frequent anatomic variations such as a hepatic artery originating in the SMA. It has been demonstrated that patients with intraoperative arterial complications have longer operative time, higher transfusion rate and more postoperative complications. Another controversial issue is the extent of lymphadenectomy in the pancreaticoduodenectomy. The randomized trials published do not recommend radical lymphadenectomy as a standard approach for pancreatic ductal adenocarcinoma.

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Pancreaticoduodenectomy (PD) is the standard surgical treatment for tumors of the pancreatic head, proximal bile duct, duodenum and ampulla, and represents the only hope of cure in cases of malignancy[1-3]. Since its initial description in 1935 by Whipple *et al*[4], this complex surgical technique has evolved and, although the mortality rate has been reduced by regionalizing interventions in high volume centers[1,3], morbidity remains high, with a rate close to 40%[5,6]. Various modifications of the classical PD have been proposed to reduce morbidity[7].

We review three key issues in PD: (1) the initial approach to the superior mesenteric artery (SMA), known as the artery-first approach; (2) arterial complications caused by anatomic variants of the hepatic artery (HA) or celiac artery stenosis; and (3) the extent of lymphadenectomy.

ARTERY-FIRST PANCREATODUODENECTOMY

It has been shown that the superior mesenteric vein (SMV) can be safely resected, the only contraindication being arterial involvement[8,9]. With the classical dissection

approach, the infiltration of the SMA is often identified at the end of the operation, obliging the surgeon to resect; this often results in a resection with positive margins[8]. The objective of PD is a R0 resection, because free margins are relevant to prognosis[10]. However, up to 20% of PD have R1 resection; the most frequently invaded margin is the peripancreatic retroperitoneal margin[11], representing 3-4 cm of tissue surrounding the origin of the SMA behind the SMV[12].

In 2003, Pessaux *et al*[13] presented a modification of the dissection of the retroportal pancreatic lamina which prioritized the dissection of the origin of the SMA, allowing complete lymphadenectomy, safe dissection of the SMA and accurate identification of anatomic variations such as a HA originating in the SMA. In 2006, the same authors[8] described a technique that they termed the "SMA-first approach", which encompasses a liberal kocherization to expose the origin of the SMA just above the point where the left renal vein crosses the aorta, and in which the dissection is started caudally along the vessel. Approximately 1-2 cm from the origin of the SMA, an anomalous right HA (RHA) may be identified; if so, it is left intact and the dissection of the SMA continues caudally to the 3rd-4th part of the duodenum. The front aspect of the SMA is dissected from the mesouncinate, at which point the invasion of the SMA can be identified and the surgery finished[8]. The origin of the superior and inferior pancreaticoduodenal arteries (IPDA) can be identified and ligated when they enter the pancreatic head and the uncinate process respectively, reducing congestion and bleeding from the pancreatic head[9].

Since the initial description, Pessaux *et al*[8] have reported several surgical techniques and approaches, all termed "artery-first". All these techniques prioritize artery dissection to identify arterial involvement, and thus assess whether the tumor is resectable before taking the irreversible step to operate[9].

Posterior approach

Pessaux *et al*[8] described the posterior approach to the SMA, indicated for resection of posteromedial tumors of the head and neck and periampullary tumors extending from the body to the head of the pancreas[9]. This procedure allows early dissection of the posterior pancreatic capsule and identification of SMA involvement and an anomalous RHA, and also facilitates *en bloc* resection of the portal vein (PV)/SMV if they are involved[1]. Its disadvantage is that it is difficult to perform in patients presenting with peripancreatic inflammation and adhesions around the pancreatic head[9], and also in obese patients[14].

Inferior supracolic/anterior approach

Hirota *et al*[15] described PD using a "no-touch isolation technique", to avoid compression of the tumor and the spread of malignant cells within the abdominal cavity. The tumor is wrapped in Gerota's fascia and the retroperitoneal margin dissected along the right side of the SMA and the abdominal aorta. The stomach can be retracted cranially to expose the pancreatic neck and, before it is sectioned, to raise its lower edge in order to assess the resectability of the tumor[9]. It is considered a useful technique for tumors of the lower edge of the pancreas and facilitates retroperitoneal dissection, especially in locally advanced tumors receiving neoadjuvant therapy[9].

"Hanging maneuver"

Pessaux *et al*[12] described an approach that combined the posterior and anterior technique, which they termed the "hanging maneuver". It has subsequently been used by other authors[11]. A tape is passed around the SMA from its origin in the aorta to its exit point in the mesentery, thus lifting up the peripancreatic retroperitoneal tissue. The traction exerted on the tape by the assistant pulls the retroperitoneal pancreatic tissue to the right, improving the exposure of the SMA and facilitating dissection at the origin of all its proximal branches, and leaving both hands of the surgeon free to control the bleeding[11,12,14]. The authors recommend this approach especially in patients with preoperative suspicion of involvement of the SMA, in patients receiving neoadjuvant therapy for locally advanced disease, in obese patients, and when the RHA originates in the SMA[12].

Inferior infracolic/mesenteric approach

Weitz *et al*[10] described the infracolic approach from the transverse mesocolon. To identify the origin of the SMA, after a modified Kocher maneuver, the small intestine is moved to the right and the peritoneum is opened parallel to the root of the mesentery on the left of the proximal jejunum and the duodenojejunal flexure. Then, the posterior part of the SMA is dissected, trying to preserve the nerves on the left side in order to avoid postoperative diarrhea. The SMV and SMA are identified from an inframesocolic position, and the dissection continued cranially. On the right side of the SMA, the anomalous or accessory RHA may be identified and the IPDA located, facilitating its early ligation and reducing bleeding[9]. Weitz *et al*[10] believe that this technique is especially useful in patients with locally advanced tumors and suspected infiltration of the SMA at the origin of the aorta or malignant tumors in the uncinate pancreas, but it is difficult to perform in obese patients and if the origin of the SMA is high.

Right/medial uncinate approach

Hackert *et al*[16] presented a modification of PD for early SMA dissection consisting of a retrograde dissection of the pancreatic head in the caudo-cranial direction. The proximal jejunum is dissected and the first jejunal loop is moved to the right of the mesenteric root in order to initiate pancreatic dissection in the uncinate process and to perform pancreatic transection in the last surgical stage. Previously, Shukla *et al*[17] described the dissection of the uncinate process from the mesenteric vessels, facilitating tumor removal and demonstrating the involvement of these vessels early during surgery. This approach is recommended in uncinate tumors and in cases with suspected involvement of the SMV or SMA[2]. Another advan-

tage of this approach is the early ligation of the IPDA, but it does not allow early identification of an anomalous RHA[9].

Left-posterior approach

Kurosaki *et al*[18] presented the "left-posterior approach" in which the superior mesenteric vessels are dissected first, clockwise from the left. This technique allows *en bloc* dissection of the superior mesenteric pedicle, provides a clear understanding of the anatomy in order to detect an aberrant RHA, and predicts the involvement of the margins in the SMA level prior to performing PD. It consists of a kocherization of the duodenum, pushing the proximal jejunum to the left and sectioning the first and second jejunal arteries at the origin of the SMA; while the proximal jejunum is pushed leftwards, the SMA rotates counterclockwise for correct dissection of the posterior and right faces of the SMA, allowing early ligation of the IPDA. The first jejunal vein is then revealed, which is the landmark for dissection; as the lower part of the pancreatic head is moved leftwards the SMV appears, the first jejunal vein is transected, and the SMV is dissected. The proximal jejunum is then moved to the right of the mesenteric pedicle allowing dissection of the connective tissue remaining on the anterior side of the SMA. This technique may be useful in tumors of the posterior part of the head of the pancreas or the uncinate process[9].

Superior approach

This approach is useful for resection of tumors on the upper edge of the pancreas or when involvement of the common HA (CHA), or of the surrounding lymph nodes, is suspected[9]. The hepatoduodenal ligament is dissected to expose the CHA and the gastroduodenal artery, with dissection from right to left to perform lymphadenectomy in this area. Subsequently the pancreas is retracted caudally, dissection proceeds caudally as far as the celiac trunk, and the origin of the SMA and the lymphatic tissue surrounding it is dissected. This technique is difficult to perform in patients presenting a low origin of the SMA[9].

Comparative studies of the different approaches

Several authors have compared these approaches with classical PD. Figueras *et al*[3] compared classical PD with the posterior artery-first approach, reporting a reduction in complications and hospital stay, while Dumitrascu *et al*[1] found no significant differences in hospital stay, early morbidity, mortality, or overall survival. Shrikhande *et al*[2] compared classical PD with the "uncinate artery-first approach" and found no evidence of significant differences in blood loss, operative time, margin involvement, lymph node yield, or complications.

Comparing conventional PD with the left posterior approach, Kurosaki *et al*[18] found no differences in operative time, blood loss or hospital stay, but reported a lower rate of recurrence and improved survival with the left posterior approach. They also recorded an increase in frequency and degree of diarrhea in patients treated with the left posterior approach, but this was controlled with antidiarrheal drugs.

Most authors agree that artery-first PD is useful in patients in whom an anomalous origin of the RHA or an accessory HA leaving the SMA is suspected. It allows early assessment of arterial involvement and thus of tumor resectability, especially in patients receiving chemotherapy and/or radiotherapy in whom tumor status is difficult to determine using computed tomography (CT), and in patients with borderline resectable disease[9].

Whichever approach is used, the standardization of "artery-first" PD will be important in reducing the number of R1 resections and in increasing survival[14].

ARTERIAL COMPLICATIONS DURING PANCREATODUODENECTOMY

Morbidity rates in PD remain high. Arterial complications are one of the possible sources of morbidity[19]. This problem has received little attention to date: some reports suggest that these complications occur only in 3%-4% of PD[19], but this seems excessively low given that various arteries around the pancreas are put at risk during dissection, arterial anomalies are frequent, and atherosclerosis can occlude arterial vessels[19]. When atherosclerosis occurs, morbidity is higher[19]. In this section, we review the most frequent arterial complications during PD: problems related to anatomical variations of the HA and celiac axis stenosis.

Hepatic artery anatomical variations

Rates of anatomical variations in the hepatic arterial system may be as high as 45%[20,21]. A thorough knowledge of HA anatomy is essential; in the presence of HA anatomical variations (HAAV), accidental ligation may occur during PD, provoking hepatic necrosis, ischemic biliary injury or an anastomotic fistula[6,20-24].

Preoperative assessment of peripancreatic vascular patterns using imaging methods is crucial for surgeons[22]. Multidetector CT is the method of choice, and multidimensional reconstruction may be very useful[6,21-23,25]. Angiography is no longer needed[20].

Several classifications of HAAV have been proposed (Covey, Hiatt, Koops and Michels)[6,20,22,23]. The most frequently described HAAV are an anomalous RHA from the SMA (10%-21%), a displaced left HA (LHA) from the left gastric artery (4%-10%), displaced RHA and LHA, an accessory RHA and/or LHA (1%-8%), a displaced CHA from the SMA or aorta (0.4%-4.5%), and quadrifurcation of the HA itself[20,23].

In the largest study carried out to date, which included 5002 abdominal CT, the crucial data regarding identification of HAAV during PD were the following: only 0.13% of patients with CHA originating in the celiac axis (normal anatomy) had a retroportal or transpancreatic course; CHA originating in the aorta always had a normal course, and CHA coming from the SMA might show different relations with the pancreas (supra, trans or infrapancreatic) and the PV and SMV (pre or retroportal and

pre or post SMV)[25].

The most important HAAV that the surgeon must bear in mind during PD are accessory RHA and displaced or accessory CHA, both arising from the SMA[23].

Displaced or accessory RHA arising from the SMA (10%-21%) is the variation that is most often identified during PD[6,20-23]. This vessel passes lateral to and behind the PV and can be felt by palpation, but it may also pass behind or through the pancreas[6,22,23]. Displaced or accessory CHA arising from the SMA, known as the hepatomesenteric trunk, is the second most frequent variation (2%-3%)[6,20] and its course is variable[20,23].

On encountering an HAAV during PD, the possible options for intraoperative management are ligation, dissection and traction away from the site of dissection, or division and anastomosis[6,20,23]: (1) the main problem with ligature of the displaced RHA is liver necrosis[20]. The ligation of accessory vessels usually has fewer clinical implications[23]. Preoperative clamping of the artery to be ligatured and post-ligature control of the flow of the non-ligated arteries is advisable[20]; (2) dissection and traction procedures are only possible in certain HAAV and tumors located in the ampulla. The procedure may be technically demanding; cancer cells may spread and there may be postoperative bleeding[20]; and (3) reconstruction of the HAAV may increase the risk of postoperative bleeding if pancreatic fistula develops[20]. Besides, there is no consensus among pancreatic surgeons regarding the desirability of arterial resections during PD.

Early in every PD, a conscious attempt should be made to define the vascular anatomy. However, in the standard approach, dissection of an HAAV coming from the SMA is usually performed late, when bleeding reduces its exposure[6]. When SMA dissection is performed first, the exposure of the HAAV is better, particularly the RHA or a CHA originating from the SMA or aorta[6,13,21].

In vessels that lie within the head of the pancreas there are several options. One is the division of the pancreas to preserve the vessel, but this is not recommended in cases of malignancy[22]. If detected preoperatively, an embolization may be performed. If identification is intraoperative, the possible technical options are ligature after temporary clamping of the vessel and checking the hepatic flow using Doppler or division and anastomosis[20,23].

In conclusion, the presence of HAAV complicates PD and their preoperative diagnosis using CT is essential. The most frequent HAAV are displaced RHA or CHA from the SMA. The artery-first approach seems to obtain a better identification of HAAV. Several technical options (ligature in the case of accessory arteries, dissection and traction or vascular reconstruction) may be performed during PD. Patients with intraoperative arterial complications have longer operative time, higher transfusion rate, and more postoperative complications[19].

Celiac artery stenosis

Celiac artery occlusion or stenosis (CAS) is frequently present (12%-50%) but it is usually of no clinical significance due to collateral pathways[26-28]. CAS has been reported in 2%-7.6% of patients undergoing PD[23]. In these patients, upper abdominal organs are at risk of necrosis from ischemia because PD resection involves the collateral vessels (the gastroduodenal and pancreaticoduodenal arteries)[19,24,26-28].

The cause of CAS may be vascular (mainly arteriosclerosis) or non-vascular: compression of the median arcuate ligament (MAL) or invasion by tumor or lymph nodes[23,26,27]. Sugae *et al*[27] proposed a morphological grading of celiac axis stenosis (A, B and C) by MAL compression according to stenosis grade and duration, distance from the aorta, and collateral pathways.

To maintain correct blood supply after PD in patients with CAS, a detailed preoperative assessment is essential[23,26,27]. The best method for defining CAS and its etiology is multidetector CT[24,27].

The treatment options are tailored to stenosis grade and etiology of CAS, but preserving collateral pathways during PD is essential[26,27]. Placing arterial preoperative stenting before PD is a valid therapeutic option especially in severe cases of CAS unrelated to MAL compression[24,26,27]. When CAS is caused by MAL compression, surgical division of the MAL is performed during PD. After division, blood flow should be restored by Doppler or palpation[23,26,27]. Gaujoux *et al*[24] only consider MAL division when the intraoperative clamping test of the gastroduodenal artery is positive. Revascularization should be performed (arterial anastomoses or bypass grafting) if MAL division does not improve perfusion, or in other selected cases[24,27].

If CAS is not diagnosed and treated during PD, there may be severe complications during the postoperative period[24]. Muros *et al*[28] showed that patients with CAS presented more serious complications (pancreatic fistula and hemoperitoneum) and more reoperations.

EXTENDED LYMPHADENECTOMY FOR PANCREATIC HEAD ADENOCARCINOMA

Pancreatic ductal carcinoma has an incidence of lymph node invasion of more than 70%[29-31]. Though many different multimodal therapy regimens have been used, long-term survival has seen little improvement, with a median survival of about 18 mo and a 5-year survival after curative resection of 6%-20%[32-34].

The multivariate analysis of Nimura's randomized study identified lymph node involvement and vascular resection as independent factors for poor prognosis[35]. A recent study also found the lymph node ratio to be a better prognostic factor than the total number of infiltrated lymph nodes.

In view of the high frequency of lymph node involvement, the high incidence of local recurrence, and the relationship between survival and node level of invasion published in some studies[34], numerous attempts have been made to increase survival by means of a more radical local resection and by extended lymphadenectomy

of the most frequently affected lymph nodes (anterior and posterior pancreaticoduodenal nodes, periaortic, and those of the SMA and the celiac trunk)[35,36]. The first report was published in 1977 by Fortner[37], who described "regional pancreatectomy" in which an extended lymphadenectomy with vascular and perineural mesenteric resection was associated with the PD. Since then, several studies have tried to increase survival with extended radical lymphadenectomy (ELA), which has been protocolized in many centers in Japan since the late 1990s. Ishikawa *et al*[38] in 1998, and Manabe *et al*[39] in 1999, published two non-randomized studies with 5-year survival rates of 28% and 33%, respectively. Several prospective non-randomized studies have been published showing a significantly higher number of lymph nodes removed in patients with ELA, but without any influence on survival. However, these studies presented higher morbidity in the form of diarrhea associated with the circumferential dissection around the SMA[40,41]. The design of these studies was heterogeneous, with different adjuvant chemotherapy regimens and different definitions of surgical radicality.

Four prospective randomized studies comparing standard lymphadenectomy (SL) *vs* extended lymphadenectomy had been published by 2005 (Pedrazzoli *et al*[32] in 1998, Yeo *et al*[29] in 2002, Nimura *et al*[30] in 2004, and Farnell *et al*[33] in 2005). All these studies applied different adjuvant chemotherapy regimens. Pedrazzoli *et al*[32] administered intraoperative radiotherapy, and Yeo *et al*[29] and Farnell *et al*[33] postoperative chemoradiotherapy. As in the previous studies, the number of lymph nodes removed was significantly higher and the operative time significantly longer with ELA.

Yeo *et al*[29] found a significantly higher rate of complications with ELA, mainly due to a greater frequency of delayed gastric emptying and pancreatic fistula (29% in SL *vs* 43% in ELA). Nimura *et al*[30] recorded severe diarrhea in 48% of patients with ELA. There was no difference in postoperative stay (range between 11 and 23 d). These randomized studies showed no significant differences in mean and long term survival between standard and extended lymphadenectomy. In-hospital mortality was similar in the two groups.

The last randomized prospective study was published by Nimura *et al*[35] in 2012, who compared a group of 51 patients with SL and a group of 50 patients with standardized extended lymphadenectomy including the lymph nodes of the hepatoduodenal ligament, CHA and mesenteric artery (both circumferentially), celiac trunk, and periaortic nodes (from the celiac trunk to the inferior mesenteric artery). In this study neither neoadjuvant nor adjuvant therapy was administered. Recruitment of patients was suspended because no survival differences were observed with ELA. The only significant differences were a longer operative time (426 min *vs* 547 min, $P < 0.0001$), a higher number of lymph nodes removed (13.3 *vs* 40, $P < 0.005$), and increased intraoperative blood loss (1118 mL *vs* 1680 mL, $P < 0.0001$) in patients with ELA. There were no significant differences in the R0 resection rate or in hospital morbidity-mortality, although the incidence of postoperative severe diarrhea was higher in the ELA group. Interestingly, tumor recurrence patterns were similar, including lymph node recurrence, although surprisingly the rate of local recurrence was higher in the group with ELA. The 1-year disease-free survival was similar, and the 5-year survival rate was 15.7% in the group with SL and 6% in the ELA group. Five-year survival in patients with negative lymph node involvement (N0) was 33.6% in SL and 15% in ELA. In patients with positive lymph node involvement (N1), survival was 6% and 0% respectively. None of these differences were significant.

CONCLUSION

In summary, randomized studies have not demonstrated a significant increase in survival with extended lymphadenectomy in patients with adenocarcinoma of the head of the pancreas. This is probably because the majority of patients have systemic disease on diagnosis, even in resectable cases, as demonstrated by the invasion of periaortic lymph nodes[6,7]. The randomized trials published do not recommend ELA as a standard approach for pancreatic ductal adenocarcinoma.

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TOPIC HIGHLIGHT

Jose Manuel Ramia, MD, PhD, FACS, *Series Editor*

Pathology handling of pancreatoduodenectomy specimens: Approaches and controversies

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Abstract

Pancreatic cancer, with a 5% 5-year survival rate, is the fourth leading cause of cancer death in Western countries. Unfortunately, only 20% of all patients benefit from surgical treatment. The need to prolong survival has prompted pathologists to develop improved protocols to evaluate pancreatic specimens and their surgical margins. Hopefully, the new protocols will provide clinicians with more powerful prognostic indicators and accurate information to guide their therapeutic decisions. Despite the availability of several guidelines for the handling and pathology reporting of duodenopancreatectomy specimens and their continual updating by expert pathologists, there is no consensus on basic issues such as surgical margins or the definition of incomplete excision (R1) of pancreatic ductal adenocarcinoma. This article reviews the problems and controversies that dealing with duodenopancreatectomy specimens pose to pathologists, the various terms used to define resection margins or infiltration, and reports. After reviewing the literature, including previous guidelines and based on our own experience, we present our protocol for the pathology handling of duodenopancreatectomy specimens.

Key words: Pancreatic ductal adenocarcinoma; Duodenopancreatectomy specimens; Resection margins; Pathology protocols

Core tip: Pancreatic cancer, one of the most lethal tumor types, is the fourth leading cause of cancer death in developed countries. The need to prolong patient survival has prompted the development of improved protocols to evaluate duodenopancreatectomy specimens and their surgical margins by pathologists. Despite the availability of several guidelines and their continual updating, there is no consensus on basic issues such as surgical margins or the definition of incomplete excision. We herein review the controversies and approaches in the literature and present our own protocol for the handling and reporting of pancreatoduodenectomy specimens by pathologists.

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INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is the most common cancer affecting the exocrine pancreas and the fourth leading cause of cancer death in both sexes in

the United States[1]. In that country, pancreatic cancer accounts for 3% of all new malignancies. It is estimated that 45220 new cases will be diagnosed there during 2013 and it will be the cause of death for 38460 patients[1]. Death rates for pancreatic cancer between 2005 and 2009 were 12.5 and 9.5 per 100000 inhabitants (males and females, respectively)[1]. In Europe, pancreatic cancer accounted for 6.2% of deaths in 2012 (78000 patients)[2]. The overall 5 year survival rate remains dismal, at around 5%[1].

Unfortunately, only 8% of pancreatic cancer patients are diagnosed in the early stages and of those, only 20% are susceptible to surgical treatment[3].

The clinical management of oncological patients relies on robust pathological data for the assessment of the extent of the disease. Despite general guidelines for the handling and pathology reporting of pancreatic specimens which are constantly updated by expert pancreatic pathologists, there is no consensus in basic terms as to what margins of surgically resected PDAC must be reported or what exactly defines an incomplete excision (R1)[4]. In this report, we review these differences in the current literature and present the protocol that is used in our institutions, based on a European trend.

PATHOLOGY MANAGEMENT OF RESECTED PANCREATIC TUMORS

One of the most important steps in pathology reporting is the dissection procedure. There is a lack of consensus, however, in the development of a standardized guide for the macroscopic management of PDAC specimens. This is perhaps due to the fact that pancreatic surgery is not performed in all hospitals so not all pathologists have access to these pathologies. In addition, the precise evaluation of resection margins has been considered less critical due to the poor prognosis of this neoplasm and its lack of response to standard chemotherapy[5].

Despite the fact that resection margin status is a key prognostic factor, the rates of microscopic margin involvement (R1) vary enormously from study to study[6-10]. The disparities may be a result of differences as to what constitutes a resection margin, the controversy over the definition of R1 status and the lack of a standardized dissection protocol of PDAC specimens[5]. In recent studies[5,11,12], an important increase in R1 resections has been reported after the use of a standardized protocol of pathological reporting of PDAC specimens. An example is given in the study by Esposito *et al*[11] in which they show a change from 14% R1 resections to 76% when a standardized protocol was applied. Other series, including our preliminary report of 2007[13], have similar changes[5,11,14].

CONTROVERSIES IN THE HANDLING OF PDAC SPECIMENS

Nomenclature of relevant margins

Four relevant margins should be studied in PDAC: (1) luminal margins (proximal gastric or duodenal and distal jejunal); (2) bile duct margin (BDM), common bile duct or common hepatic duct margin; (3) pancreatic transection margin (PTM); and (4) pancreatic circumferential or radial margin (CRM).

The first three margins are universally accepted and easily recognizable in the specimen. In addition, the BDM and PTM can be examined intraoperatively.

According to Verbeke's reports, the CRM can be divided anatomically into an anterior surface or pancreatic anterior margin (PAM) and a posterior surface or pancreatic posterior margin (PPM). They are separated by a pancreatic medial margin (PMM), the part of the surface of the pancreatic head that faces the superior mesenteric (SM) vessels[5,15].

The PAM cannot be considered a true margin since there is no transection by the surgeon at this level. Although the PPM and PMM are truly the most important margins since they are frequently affected[5,12,13,16], we cannot ignore the fact that the presence of tumor cells on the anterior surface is likely to increase the risk of local tumor recurrence[5,17].

The PMM refers to the area that faces the superior mesenteric vessels, totally or partially surrounding the superior mesenteric vein. It has a shallow groove-like shape and a slightly glistening surface flanked by ties. Segments of vessels can be found when involved in the cancer[5]. The PMM is the margin most frequently involved and therefore requires careful assessment[15,18-20]. The PMM has many names, such as "vascular bed", "uncinated process margin", "mesenteric margin" or even "retroperitoneal margin". The last denomination may cause confusion[5,13,16] given that the entire head of the pancreas and not just this surface is located in the retroperitoneum.

The PPM is the area adjacent to the superior mesenteric artery the surgeon transects so it is a true margin[5].

In a recent publication by Khalifa *et al*[16], the nomenclature commonly used for pancreatic margins is reviewed. It makes evident the great variability, especially that in relationship to the circumferential margin, and the need for consensus. The terms "posterior" and "medial" margins are commonly used by European pathologists[11,16,21], while "deep retroperitoneal posterior surface" or "uncinated process" margins are the terms chosen by the College of American Pathologists (CAP) and the American Joint Committee on Cancer (AJCC)[22-24] (Figure 1).

Differences in dissection protocols

A wide range of different dissection techniques are used given the lack of consensus. Many of them are based on tradition rather than on an evidence-based rationale[5].

For many years, the longitudinal opening of the main pancreatic and biliary duct has been the standard technique used by European and American pathologists[15,18-21,25-27]. This method, however, interferes with the CRM assessment and is uninformative since the majority of PDAC do not arise in the main duct, with the exception of the intraductal papillary mucinous neoplasm[5].

In some classic American protocols, there is no speci-

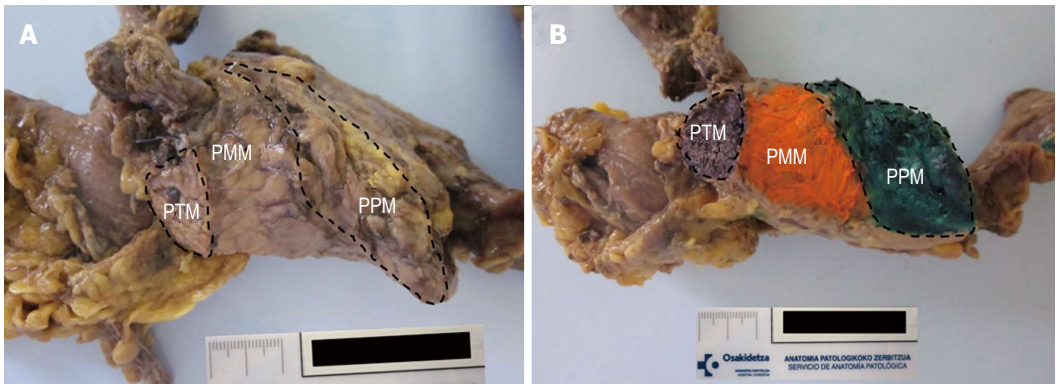

Figure 1 Pancreatoduodenectomy specimen images. A: Pancreatoduodenectomy specimen after fixation (posterior view); B: The circumferential soft tissue margins were inked (PTM: Violet, PMM: Orange, PPM: Green). PTM: Pancreatic transection margin; PMM: Pancreatic medial margin; PPM: Pancreatic posterior margin.

fied procedure for the specimen dissection[28] and the need to ink some of the margins and submit them is only superficially addressed[19,22,23,29].

Methods based on sections parallel to the pancreatic major axis, including a longitudinal section of the duodenal wall, have been used in Europe for many years[25]. The resulting sections are too thick and comprise different planes, something which makes it difficult for the pathologist to reconstruct the specimen or assess tumor size and margin status[5].

Both the *Armed Forces Institute of Pathology* (AFIP) in its 3rd edition[27] and Allen and Cameron in 2004[30] suggested a way of handling specimens based on the opening of biliary and pancreatic ducts with sections perpendicular to the ducts. Recently, in their 4th edition, the AFIP[31] recommended performing perpendicular sections to the main duct. That notwithstanding, these sections would be tangential to the duodenal wall, thus making the analysis of the ampulla, distal pancreatic and bile duct difficult[5].

The Japan Pancreas Society[32] has suggested slicing perpendicular to an axis that follows the curvature of the pancreatic head, even although the constant change of planes is an inconvenience[5].

The procedure performed by Westgaard *et al*[12] consists of inking the retroperitoneal margin, performing a 5-10 mm thick section parallel to this margin and serially slicing perpendicular to the ink.

In the last few years, a new standardized dissection technique[5,11,15,33] has been developed in Europe, especially in the United Kingdom. It is characterized by a serial slicing of the entire pancreatic head in a plane perpendicular to the longitudinal axis of the duodenum which avoids opening the biliary or pancreatic duct (Figure 2). The advantage of this method is its simplicity. There is no dependency of location or nature of the disease and a great number of sections are produced. This permits an extensive study of the lesion and its relationship with anatomical structures and surgical margins[5,15].

Differences in international protocols

The AJCC and CAP protocols recommend inking and cutting sections through the tumor at its closest approach to the retroperitoneal margin of the uncinate process (uncinate margin) and retroperitoneal posterior surface[22,23].

Only Allen and Cameron[30] recommend the need for analyzing the following margins in their book: superior, inferior, capsular anterior, posterior retroperitoneal and medial (superior mesenteric vein).

The Royal College of Pathologists[21] includes the transection margins (gastric, duodenum, pancreatic and common bile) and the dissected margins (superior mesenteric vessels and medial and posterior margins) in their histopathological report.

The anterior surface of the pancreas is not a true surgical margin but invasion of this surface has been associated with local relapse and decreased survival times[17,34]. For this reason, some authors and guides suggest reporting this margin[5,11,21,31], although it is not reported by the CAP[22] or by the 7th edition of the AJCC[23].

Margin involvement: R1 status

The lack of consensus on margins not only affects their nomenclature and inclusion in the pathological report, but also the definition of R1.

The role of margin involvement and its prognostic relevance has been well characterized in other cancer types, such as rectal cancer. Verbeke, though, states that *"margin status in pancreatic cancer has been neglected"*[5].

Resection margin involvement (R1) seems to be an important prognostic factor in pancreatic cancer but R1 rates reported in the literature vary enormously. Rates as disparate as 16% and > 75% have been reported in different studies and consequently clinical outcome correlation has been observed in some but not all[5,6,15,35].

For the majority of American pathologists, there is a positive margin (R1) only when the tumor is directly in contact with the inked margin (0 mm clearance)[13,16,22,31,35]. For European pathologists, R1 margin involvement is established when the distance between the tumor and the resection margin is 1 mm or less[5,11,12,15,21]. This is called the "1 mm rule" and was taken from the R1 definition of rectal cancer assessment[21].

Another confusing circumstance is when there is no

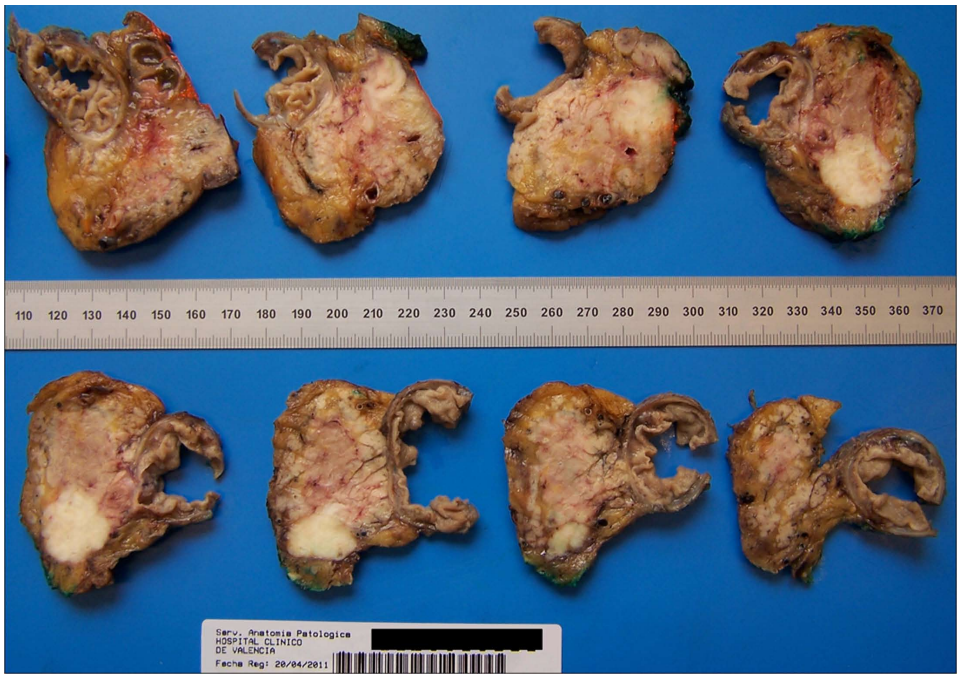

Figure 2 Consecutive parallel sections of 0.5 cm thickness following an axial plane perpendicular to the duodenal axis. Tumor seems to be in contact with the inked margin.

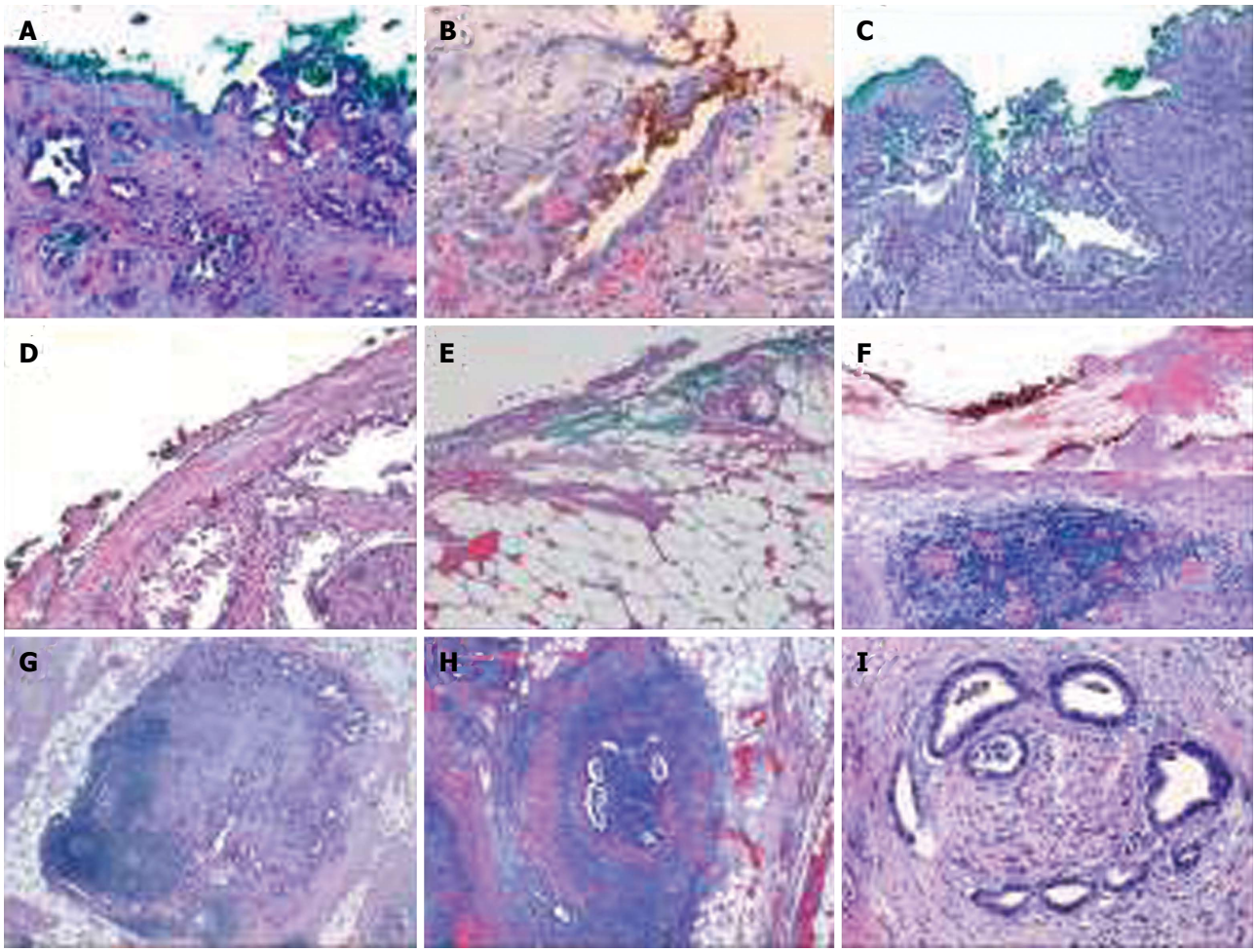

Figure 3 Microscopic picture. A-C: Microscopic picture of tumor glands in direct contact with an inked margin (R1 resection) (HE × 200, × 400 and × 200, respectively); D: Neoplastic cells within 1 mm of the resection margin colored in black (HE × 200); E, F: Examples of free medial or posterior margin (HE × 200); G: Ganglionar metastases (HE × 200); H: Vascular invasion (HE × 200); I: Perineural invasion (HE × 400).

PATHOLOGIC REPORT OF PANCREATIC CARCINOMA AT H.C.U.VALENCIA[1]

Name: _____ Age: _____
Case number: _____ Date: _____

Specimen type:
- ☐ Cephalic duodenopancreatectomy
- ☐ Cephalic duodenopancreatectomy with pyloric preservation
- ☐ Total pancreatectomy
- ☐ Distal pancreatectomy
- ☐ Central pancreatectomy

Tumor size: ____ x ____ x ____ cm

Macroscopic characteristic:
- ☐ Solid
- ☐ Cystic
- ☐ Polypoid
- ☐ Other: _____

Histologic type:[2]
- ☐ Ductal adenocarcinoma
- ☐ Adenosquamous carcinoma
- ☐ Other: _____

Histologic grade:[3]
- ☐ Well differentiated (G1)
- ☐ Moderately differentiated (G2)
- ☐ Poorly differentiated (G3)
- ☐ Undifferentiated (G4)
- ☐ Others: _____

Invasion:
- ☐ Vascular
- ☐ Lymphatic
- ☐ Perineural

Tumor site:
- ☐ Pancreatic head
- ☐ Pancreatic body
- ☐ Pancreatic tail
- ☐ Uncinate process
- ☐ Duodenal ampulla
- ☐ Other:_____

Tumor extension:[4]
- ☐ Carcinoma in situ (pTis)
- ☐ Limited to the pancreas ⩽ 2 cm (pT1)
- ☐ Limited to the pancreas >2 cm (pT2)
- ☐ Extends beyond the pancreas (pT3)
- ☐ Celiac axis or SMA involvement (pT4)

Precursor lesions:
- ☐ PanIN
- ☐ IPMN
- ☐ Others: _____

Non neoplastic lesions:
- ☐ Bile duct obstruction
- ☐ Pancreatic duct obstruction
- ☐ Pancreatic calculi
- ☐ Chronic pancreatitis
- ☐ Others: _____

Treatment effect (neoadjuvant therapy):[5]
- ☐ Complete response (grade 0)
- ☐ Moderate response (grade 1)
- ☐ Minimal response (grade 2)
- ☐ Poor response (grade 3)

Posterior circumferential margin (retroperitoneal)
- ☐ Uninvolved (> 1 mm)
- ☐ Involved
  - ☐ Direct: tumor in contact with inked margin
  - ☐ Direct: tumor ⩽ 1 mm (specify distance:_____)
  - ☐ Indirect (vascular, lymphatic or perineural) ⩽ 1 mm
  - ☐ Indirect lymph node metastasis ⩽ 1 mm

Medial circumferential margin (vascular)
- ☐ Uninvolved (> 1 mm)
- ☐ Involved
  - ☐ Direct: tumor in contact with inked margin
  - ☐ Direct: tumor ⩽ 1 mm (specify distance:_____)
  - ☐ Indirect (vascular, lymphatic or perineural) ⩽ 1 mm
  - ☐ Indirect lymph node metastasis ⩽ 1 mm

Bile duct margin: ☐ Uninvolved ☐ Involved

Pancreatic transection margin: ☐ Uninvolved ☐ Involved

Status Margins: ☐ R0 ☐ R1

Lymph node metastases: ☐ Nx ☐ N0 ☐ N1 Distance metastases: ☐ Mx ☐ M1

Lymph nodes	+	Total	Lymph nodes	+	Total
Peripancreatic (station 13, 17, 18)			Celiac (station 9)		
Suprapyloric (station 5)			Hepatoduodenal ligament (station 12)		
Infrapyloric (station 6)			Others:____		
Left gastric artery (station 7)			TOTAL		

Comments:

Explanatory notes:

1. This protocol is used for exocrine pancreatic and periampullary tumors.
2. Histologic types according to the WHO classification[51]
3. Differentiation grades (applicable only to ductal adenocarcinoma)[23]

Grade 1	Well differentiated	> 95% of tumor composed of glands
Grade 2	Moderately differentiated	50%-95% of tumor composed of glands
Grade 3[1]	Poor differentiated	5%-49% of tumor composed of glands
Grade 4[2]	Undifferentiated	< 5% of tumor composed of glands

- [1]Signet-ring cell carcinoma is considered grade 3
- [2]Undifferentiated (anaplastic) carcinoma is considered grade 4
- Other types are not graded.

4. Primary tumor (TNM classification)[23]

Tis	Carcinoma *in situ*
Pancreas	
T1	Tumor limited to the pancreas, 2 cm or less in greatest dimension
T2	Tumor limited to the pancreas, more than 2cm in greatest dimension
T3	Tumor extends beyond the pancreas, but without involvement of the celiac axis or the superior mesenteric artery
T4	Tumor involves the celiac axis or the superior mesenteric artery (unresectable primary tumor)
Ampulla of Vater	
T1	Tumor limited to ampulla of Vater or sphincter of Oddi
T2	Tumor invades duodenal wall
T3	Tumor invades pancreas
T4	Tumor invades peripancreatic soft tissues, or other adjacent organs or structures
Distal extrahepatic bile duct	
T1	Tumor confined to the bile duct
T2	Tumor invades beyond the wall of the bile duct
T3	Tumor invades the gall bladder, liver, pancreas, duodenum or other adjacent organs
T4	Tumor involves the celiac axis or the superior mesenteric artery

5. Treatment effect (applicable to carcinomas treated with neoadjuvant therapy)[52]

No viable tumoral cells	Complete response (grade 0)
Single cells or small groups of tumoral cells	Moderate response (grade 1)
Residual tumor with fibrosis	Minimal response (grade 2)
Extensive residual tumor	Poor (grade 3)

Figure 4 Elaborated checklist for the pathological reporting of pancreatic ductal adenocarcinoma.

direct margin involvement by the tumor. Despite the absence of clear evidence, The Royal College of Pathologists suggests considering the incomplete excision to be an R1 resection if lymph node metastases or perineural/lymphovascular invasion is within the 1 mm limit (indirect invasion of R1)[5,11,21]. Conversely, according to the tumor-node-metastasis staging system of the AJCC, the resection margin is considered R1 indirectly only when tumor cells are attached to or invade the vessel wall[36] (Figure 3).

Lymph node metastases

Lymph node metastases (N1) have been shown to be an independent negative prognostic factor in multivariate analysis[10,37-41]. Nevertheless, the lymph node ratio, defined as the ratio of the number of positive lymph nodes to the total number of lymph nodes evaluated, is now considered a more powerful prognostic marker than the overall nodal status in resected pancreatic cancer[10,13,42-48].

In the 5th edition of the AJCC[49], N1 was subdivided into 2 categories, N1a and N1b, depending on the number of lymph nodes affected (3 or less for N1a and more than 3 for N1b). In the subsequent versions (6th and 7th), this subdivision was changed. They considered N1 to be lymphatic metastases no matter how many lymph nodes were involved[23,29,49]. The following lymph nodes were considered to be regional: hepatic artery nodes, superior mesenteric artery nodes, retroperitoneal and lateral aortic nodes, infrapyloric and subpyloric nodes for tumors in the head; and celiac, pancreaticolieno and splenic nodes for tumors arising in the body and tail[22]. Tumor involvement of other nodal groups is considered distant metastasis[50]. In the Japan Pancreas Society, lymph node stations are classified into groups designated by numbers[32].

According to the CAP, the optimal histological examination should include a minimum of 15 lymph nodes[22,40]. This number is an indicator of the quality of the surgical procedure and pathological handling.

Direct extension of the primary tumor into lymph nodes is classified as lymph node metastasis[22,51].

HANDLING AND REPORTING PROTOCOL OF PDAC AT HOSPITAL CLÍNICO UNIVERSITARIO, VALENCIA, SPAIN

Following the published reports and guidelines, we have elaborated on a checklist for the pathological reporting of PDAC at our institution[53] based on the Verbeke reports (Figure 4).

We propose the following steps for the dissection protocol: (1) leave the specimen for 24-48 h in formaldehyde for the correct fixation after opening through the antimesenteric border of the duodenum; (2) explore the pancreatic anatomy in order to identify the different parts (head, body and tail) and give it the correct orientation in readiness for dissection. Identify the margins (circumferential resection margin composed of the PAM, PPM and PMM and the pancreatic transection margin, or PTM); (3) ink the margins indicated in step 2 in different colors; (4) slice the luminal margins (proximal gastric or duodenal and distal jejunal), BDM, common bile duct or common hepatic duct margin and PTM; (5) analyze the gastro-intestinal lumen to identify any ampullary or other lesions; (6) following the European guidelines, slice the entire pancreatic head in a plane perpendicular to the longitudinal axis of the duodenum through the center of the ampulla. Identify the tumor, its size and relationships to structures and its distance to the margins; (7) continue slicing in parallel sections with a thickness of 5 mm in order to have samples of the tumor that show its relationship with the different anatomical structures (duodenum wall, ampulla) and inked resection margins; (8) separate a sample of non-neoplastic pancreas; and (9) identify lymph nodes from the different stations for individual analysis.

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TOPIC HIGHLIGHT

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Role of endoscopic ultrasound in the diagnosis of pancreatic cancer

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Abstract

Endoscopic ultrasonography (EUS) with or without fine needle aspiration has become the main technique for evaluating pancreatobiliary disorders and has proved to have a higher diagnostic yield than positron emission tomography, computed tomography (CT) and transabdominal ultrasound for recognising early pancreatic tumors. As a diagnostic modality for pancreatic cancer, EUS has proved rates higher than 90%, especially for lesions less than 2-3 cm in size in which it reaches a sensitivity rate of 99% *vs* 55% for CT. Besides, EUS has a very high negative predictive value and thus EUS can reliably exclude pancreatic cancer. The complication rate of EUS is as low as 1.1%-3.0%. New technical developments such as elastography and the use of contrast agents have recently been applied to EUS, improving its diagnostic capability. EUS has been found to be superior to the recent multidetector CT for T staging with less risk of overstaying in comparison to both CT and magnetic resonance imaging, so that patients are not being ruled out of a potentially beneficial resection. The accuracy for N staging with EUS is 64%-82%. In unresectable cancers, EUS also plays a therapeutic role by means of treating oncological pain through celiac plexus block, biliary drainage in obstructive jaundice in patients where endoscopic retrograde cholangiopancreatography is not affordable and aiding radiotherapy and chemotherapy.

Key words: Endosonography; Pancreatic neoplasms; Endoscopy; Diagnosis; Neoplasm Staging; Therapeutics

Core tip: In this article, the role of endoscopic ultrasonography as a diagnostic, staging and therapeutic procedure in patients with pancreatic cancer is discussed and all the current knowledge on this subject is summarized, providing the reader with a quick update.

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INTRODUCTION

Pancreatic cancer is the fourth leading cause of cancer-related death in men and the first leading cause in women, with an approximate incidence of ten per 100000 population per year[1,2].

Multiple-imaging modalities are used in combination in the diagnosis and staging of pancreatic cancer: transabdominal ultrasound, computed tomography, magnetic resonance and endoscopic retrograde cholangiopancreatography (ERCP).

The prognosis of pancreatic cancer is dismal, with a 1 and 5 year survival rate at all stages at diagnosis of 24% and 5%, respectively, according to the latest from the American Cancer Society[3]. Without treatment, the average survival of patients with pancreatic cancer is four months[4]. Endoscopic ultrasound (EUS) could be a good imaging technique for a better selection of patients for an effective curative treatment.

In addition, by the time pancreatic cancer manifests symptoms that demand medical attention, it has already spread to the point of unresectability in nearly 80%-90% of patients because of metastatic disease[4,5]. It is especially in these patients where the therapeutic spectrum of EUS is growing. Treatment of oncological pain through celiac plexus block, biliary drainage in obstructive jaundice in patients where ERCP is not affordable and aiding radiotherapy and chemotherapy are some examples of this.

Therefore, EUS has several roles in the widespread sphere of pancreatic cancer. The introduction of EUS in the 1980s was received with great enthusiasm because of the improved information it could provide on the pancreas by overcoming the limitations associated with the use of transabdominal ultrasound. EUS with or without fine needle aspiration (FNA) has been shown to be a cost-effective technique for evaluating pancreatobiliary disorders, particularly where others have failed[6], and has a higher diagnostic yield than positron emission tomography (PET), computed tomography (CT) and transabdominal ultrasound for recognizing early pancreatic tumors[1,2].

Pancreatic cancer diagnosis can be made with accurate sensitivity and specificity by EUS because of its inherent advantage of a high-frequency transducer placed in close proximity to the tumor which provides a high resolution image, especially with the incorporation of contrast enhanced images in the last years, making possible a differential diagnosis with other pathologies, such as chronic pancreatitis and neuroendocrine tumors[7], and a histological confirmation using EUS-FNA

THE ROLE OF EUS FOR DIAGNOSIS OF PANCREATIC CANCER

Numerous studies indicate that EUS is highly sensitive for the detection of pancreatic tumors with rates higher than 90%[8], especially for lesions less than 2-3 cm in size in which it reaches a sensitivity rate of 99% *vs* 55% for CT[9,10]. Although the sensitivity for tumor detection is high, it is also important to note that it has a very high negative predictive value (NPV)[11,12]. This is quite important for clinicians because it means that EUS could reliably exclude pancreatic cancer. On the other hand, this evidence comes from one study only and certain conditions explained further on in the text may hinder a diagnosis of pancreatic cancer.

EUS also has the ability to provide FNA which has made it essential in the evaluation of patients with solid pancreatic lesions since most patients require a tissue diagnosis before treatment.

Certain tumor extrinsic conditions exist that may hinder the identification of pancreatic cancer[13]: chronic pancreatitis with a severe inhomogeneous echotexture, diffuse infiltration by tumor, prominent ventral/dorsal division and acute pancreatitis lasting less than 4 wk.

THE ROLE OF EUS IN THE DIFFERENTIAL DIAGNOSIS OF PANCREATIC CANCER

Differential diagnosis of solid pancreatic masses remains a challenge. Dynamic contrast-enhanced CT is the most widespread imaging technique for this purpose and has been considered the most comprehensive tool for diagnosis and surgical staging of pancreatic malignancies[5]. Despite all the advances with the multidetector helical CT scan, differential diagnosis between mass-forming chronic pancreatitis, ductal adenocarcinoma and autoimmune pancreatitis based on only CT image is still difficult[14,15].

Magnetic resonance imaging (MRI) could also be useful in the differentiation of pancreatic solid masses but several studies have demonstrated that is less sensitive than CT and EUS[16,17]. The administration of secretin during magnetic resonance cholangio-pancreatography can be useful, enhancing the image of the main pancreatic duct, providing pancreas function and duct shape information as dilation[18].

Currently, ERCP has no clinical role in the diagnosis and staging of pancreatic cancer. Indirect findings such as combined dilation of the bile and the pancreatic duct or abrupt cutoff in the main pancreatic duct or a solitary long stricture of the pancreatic duct could raise suspicion of malignant disease but may also be observed in chronic pancreatitis.

PET is an image modality which relies upon detection of functional activity rather than lesion size alone. Tumors have enhanced glucose uptake and normal pancreas has low glucose utilization rate, fluorodeoxyglucose labelled with radioactive fluorine (^{18}FDG-PET) readily accumulates in malignant cells and can be detected by a PET camera[19]. However, the role of ^{18}FDG-PET in evaluation of primary pancreatic adenocarcinoma has not been established in evaluating tumor response to neoadjuvant chemoradiotherapy or in the evaluation of recurrent disease after surgical resection.

EUS is considered to be one of the most accurate methods for diagnosis of inflammatory, cystic and neoplastic diseases of the pancreas[4,20,21] and recent studies recommend it for the differential diagnosis of solid pancreatic masses, although accuracy in differentiation between benign inflammatory masses and malignant tumors of pancreas has not been higher than 75%[22-27].

In a study by Eloubeidi *et al*[28], 101 patients with solid pancreatic masses underwent an average of 4 needle passes with EUS-FNA, resulting in a sensitivity of 95%, specificity of 95%, positive predictive value (PPV) of 100% and NPV of 85.2%.

EUS-FNA can be made using different types of

needles. Small calibre needles (25 G) have a similar cytology yield as large calibre needles (19 G) with less blood contamination and the advantage of greater flexibility for difficult-to-reach areas such as the uncinate process[29]. The prospective study by Sakamoto *et al*[30] showed that 25-gauge was the best choice of needle for cytological diagnosis of solid pancreatic lesions and, in cases in which a histological diagnosis is desired, the 22-gauge FNA needle and 19-gauge trucut needle may be an advantage in head/uncinate and body/tail lesions, respectively.

On-site cytopathology for some investigators is deemed a superior standard of care with the provision of opportunity for real-time interpretation of samples[31,32] so that it improves the diagnostic yield of EUS-FNA independent of the number of needle passes undertaken for tissue sampling[33]. If this cannot be provided, 5-6 passes for pancreatic masses and 2-3 passes for peripancreatic lymph nodes and metastases will provide the maximum yield[34]. Also, having an experienced cytopathology technician or to specifically train a EUS nurse to prepare and determine cellular adequacy for each sample[33] is helpful in these cases. In cases in which initial cytology is indeterminate or non-diagnostic, the literature supports reattempting EUS-FNA and combining routine cytology with fluorescence in situ hybridization (FISH) and K-ras/p53 analysis to improve the diagnostic yield. This combination yields 87.9% sensitivity, 93.8% specificity, 96.7% PPV, 78.9% NPV and 89.8% accuracy in the Reicher and colleagues retrospective study[35]. FISH plus K-ras analysis correctly identified 60% of atypical FNAs with a final malignant diagnosis.

EUS is considered a safe procedure with complication rates as low as 1.1%-3%[36]. Commonly reported complications include bleeding (1%-4%), pancreatitis (1%-2%), perforation (0.03%)[37] and rarely tumor seeding after EUS-guided FNA[38-42]. The risk of tumor seeding along the needle tract has been a concern especially in Japan. Although the reported incidence of tumor seeding after EUS-FNA is scarce, the indication of EUS-FNA for small lesions located at pancreas body/tail where the aspiration route will not be included in the resection area needs to be carefully considered. When pancreatic head lesions are evaluated by FNA, there is a theoretical risk of cancer seeding, but this has never been reported after EUS-FNA because after a Whipple procedure, the potential sites of seeding are removed. As for patients with unresectable disease, most die of disease progression before any seeding is detected. If the decision is to proceed to EUS-FNA, patients must be fully aware of the remote risk of seeding to the gastric wall[39]. There are two cases of tumor seeding along a EUS-FNA tract in pancreatic adenocarcinoma and both were pancreatic tail adenocarcinomas[39,40]. The only other two reports related to tumor seeding after EUS-FNA were peritoneal dissemination after EUS-FNA of pancreatic intraductal papillary mucinous neoplasia[41] and metastatic melanoma[42]. Whether this risk is increased by the needle size or number of passes remains uncertain.

The sensitivity of EUS-FNA for malignancy in patients with chronic pancreatitis is lower compared to when the surrounding parenchyma is normal[27,43-47]. Studies by Fristcher-Ravens *et al*[27] and Varadarajulu *et al*[44] found a sensitivity of 54% and 73.4% in parenchymas with chronic inflammation *vs* 89% and 91.3% in normal parenchyma respectively ($P = 0.02$). A systematic review of 53 studies estimated a NPV of EUS-FNA in the diagnosis of pancreatic adenocarcinoma as 60%-70%[48] which makes a new function mandatory in cases where the first EUS-FNA has been benign. The Procore® histology needle has been designed in order to optimize tissue sampling of EUS-FNA, allowing a histological evaluation with an overall accuracy of 89.4% in solid pancreatic lesions[49].

Recently, quantitative EUS elastography (QE-EUS) has been developed in an attempt to make the elastography interpretation less subjective than the old qualitative EUS-elastography. In the Iglesias-Garcia *et al*[23] study with 86 patients with solid pancreatic masses, the strain ratio (ratio of elasticity in the target area over soft referent tissue) was significantly higher among patients with malignant pancreatic tumors compared to those with inflammatory masses. Normal tissue showed a mean strain ratio of 1.68 (95%CI: 1.59-1.78), inflammatory masses 3.28 (95%CI: 2.61-3.96) and pancreatic adenocarcinoma 18.12 (95%CI: 16.03-20.21) ($P < 0.001$). The sensitivity and specificity of the strain ratio for detecting pancreatic malignancies in solid masses using a cut off value of 6.04 were 100% and 92.9% respectively, higher rates than obtained with qualitative elastography (100% and 85.5% respectively)[50].

Contrast-enhanced EUS (CEH-EUS) is performed with the application of contrast agents. Numerous US contrast agents (UCAs) are commercially available. Levovist®, the first agent for general use, is made of a galactose microcrystal filled with air bubbles which, shattering under a high sound pressure, emits pseudo-Doppler signals. With the development of second UCAs (Sonovue® and Sonazoid®) which contain inert gases with low solubility in water, the stability and duration of the contrast and real-time vascular images have been increased. The risk for drug allergy is small because of the small molecular weight of microbubbles and they are also applicable for patients with liver and renal dysfunctions because it is excreted by exhalation[51,52]. Most carcinomas, neuroendocrine tumors and inflammatory pseudotumors are simply depicted as hypoechoic masses, but the use of contrast agents in EUS has been shown to improve the characterization of the vasculature inside the organ of interest, to better delineate such hypoechoic masses. According to published reports, hypoenhancing masses were regarded as a sign of malignancy in CEH-EUS. The first feasibility study reported good values of sensitivity, specificity and accuracy for the differential diagnosis between adenocarcinoma and focal chronic pancreatitis[53]. This was further confirmed in two other studies by Sakamoto *et al*[54] and Dietrich *et al*[55] in which adenocarcinomas showed hypoenhancement compared with neuroendocrine tumors and pseudotumoral (mass-forming) pancreatitis, which

showed isoenhancement or hyperenhancement. Fukusawa *et al*[56] reported a prospective study, concluding that in most cases of pancreatic adenocarcinoma, CEH EUS exhibits a hypoperfusion pattern compared with the adjacent normal pancreatic tissue, whereas autoimmune pancreatitis/chronic pancreatitis exhibits iso-perfusion and pancreatic neuroendocrine tumors (PNET) exhibit a hyperperfusion pattern[56]. Fusaroli *et al*[57] found that a hypoenhancing mass with a inhomogeneous pattern diagnosed pancreatic adenocarcinoma with a sensitivity of 96% and more accuracy than standard EUS. Hyper-enhancement specifically excluded adenocarcinoma (98%), although with a low sensitivity. Seicean *et al*[58] introduced the use of quantitative CEH-EUS for differential diagnosis between pancreatic cancer and chronic pancreatitis, with the index of contrast uptake lower in adenocarcinoma compared to cases with mass-forming chronic pancreatitis. Also, using pulsed Doppler could help with the differential diagnosis between adenocarcinomas and chronic pseudotumoral pancreatitis. Pancreatic adenocarcinomas show mainly arterial-type signals and chronic pseudotumoral masses show both arterial-type and venous-type signals[59]. The first meta-analysis that summarized the available evidence of the diagnostic performance of CEH-EUS for the differential diagnosis of pancreatic adenocarcinomas showed that CEH-EUS had a pooled sensitivity of 94% (95%CI: 91-95) and a pooled specificity of 89% (95%CI: 85-92), so finding a hypoenhancing lesion was a sensitive and accurate predictor of pancreatic adenocarcinoma[60]. The variation in this study in comparison with Fusaroli *et al*[57] may have occurred because more patients with severe chronic pancreatitis were enrolled in the Fusaroli *et al*[57] study, which may have altered the enhanced pattern of pancreatic adenocarcinomas. Severe forms of chronic pancreatitis mean less intense intralesional "parenchymographic" enhancement and fibrosis resulting in decreasing vascular flow[62-64]. Iglesias-Garcia *et al*[65] compared the aforesaid QE-EUS to CEH-EUS. The authors concluded that the diagnostic accuracy of QE-EUS in pancreatic masses is superior to CEH-EUS and, furthermore, that addition of CEH-EUS does not significantly increase the diagnostic accuracy of QE-EUS.

THE ROLE OF EUS IN STAGING OF PANCREATIC CANCER

Surgery is the only curative treatment for pancreatic cancer. Statistics for survival in pancreatic cancer, where 5 year survival rates are as low as 10%-25% after a successful surgery[66,67], have been changing because of identification of appropriate candidates for surgery by a good staging, approaching a 5 year survival rate of 40% if margins and nodes are negative and the resection is made by experienced surgeons[68,69].

However, even with the newest diagnostic workup, pancreatic cancer at laparotomy is often found to be more advanced than originally thought[70,71].

Currently, the preferred modality for pancreatic cancer staging and assessing resectability is CT because its low cost and high availability[72] and MRI for preoperative assessment of pancreatic cancer, with an accuracy of 86% *vs* 71% even with comparable sensitivity of MRI for detecting pancreatic cancer (88%-96%)[73].

EUS has been found to be superior to the recent multidetector CT (MDCT) for T staging[74-77], with less risk of overstaying in comparison to both CT and MRI[78] so that patients are not being ruled out of a potentially beneficial resection. In a recent study, the sensitivity of EUS was higher than MDCT but MDCT was more specific, especially in the assessment of vascular invasion. The correct decision could be achieved in 63% in patients with either MDCT or EUS, in 9% of patients with EUS alone and in 14% of patients with MDCT alone, but the success rate rises to 86% when they are used in combination[79].

The accuracy for N staging with EUS is 64%-82%[80]. Only one study found that EUS is also better than CT for N-staging (93.1% *vs* 87.5% respectively), but most of the studies have found no difference between CT and EUS in predicting resectability in relation to node involvement[74,78-81]. Criteria for the identification of lymph node metastasis are used in different studies: spherical shape, hypoechoic node, well delineated boundaries and 10 mm diameter or more. These criteria normally are not enough and EUS-FNA is often required.

EUS has been found to be better at peripancreatic and periceliac lymphadenopathy detection (87.5%), and vascular infiltration (90%), especially for mesenteric vessels that also have a higher ability to correctly predict surgical resectability[82-84]. EUS has shown a good ability to detect vascular invasion, showing low sensitivity in the superior mesenteric artery (17%) and celiac artery (50%), although the portal venous system was correctly assessed by EUS in 95% of cases, compared with angiography (85%) and CT (75%)[85,86]. However, differently from radial EUS, linear EUS can show arterial vessels longitudinally using a linear image and both the celiac and superior mesenteric arteries are easily followed from the stomach. A recent prospective study by Tellez-Avila *et al*[87], in which the accuracy of linear-EUS and CT to determinate vascular invasion is evaluated in 50 patients with pancreatic cancer, EUS is a very good option to detect vascular invasion and is especially sensitive for arterial invasion (PPV EUS 100% *vs* PPV CT 60%).

Tumor conditions may also affect the accuracy of EUS staging[88], such as peritumoral inflammatory changes and attenuation of ultrasound beam in large tumors. For this reason, tumors smaller than 3 cm in size are more accurately staged with EUS.

THE ROLE OF EUS AS PALLIATIVE TREATMENT OF PANCREATIC CANCER: THERAPEUTIC OPTIONS

In patients with advanced unresectable disease, chemotherapy, radiation or a combination of both may positively influence overall survival and quality of life. The

therapeutic spectrum of EUS has turned endoscopy into an integral component of palliative treatment in patients with inoperable disease. EUS offers access to lesions in different parts of the pancreas, including anatomical regions that are difficult to approach percutaneously.

CELIAC PLEXUS NEUROLYSIS

Pain is one of the most prevalent symptoms in pancreatic cancer at presentation (75%) and its incidence increases as the disease advances to more than 90% of patients[89]. Pain control is the main therapeutic goal for clinicians in palliative care of pancreatic cancer patients and the conventional management with high doses of narcotics and the inherent adverse effects may further impair quality of life[90-92].

Before 2010, celiac plexus neurolysis (CPN) was considered an effective technique for controlling pain and reducing narcotic requirements in patients with pancreatic cancer[89-93]. However, a recent meta-analysis of five randomised controlled trials documented a fair response to CPN with an overall reduction in the visual analog pain scores[89]. A recent systematic review that aimed to determinate its efficacy and safety in reducing pancreatic pain found that the statistical evidence of the superiority of CPN over analgesic therapy or reducing opioid use was weak[94,95]. On the other hand, a recent randomised trial of early EUS guided CPN concluded that early EUS-CPN provides better pain relief in patients with painful, inoperable pancreatic adenocarcinoma and may prevent progressive increases in morphine consumption compared with conventional management, especially in patients who do not receive chemotherapy and/or radiation therapy, so they recommend it to be considered during diagnostic and staging EUS in all patients with predicted survival of several months where a confirmation of painful, locoregional and inoperable pancreatic cancer is obtained[95]. Despite better pain control, early EUS-CPN did not produce a demonstrable improvement in quality of life, but this was not a study powered to look for effects on quality of life.

BILIARY DRAINAGE

EUS-guided biliary drainage (EUS-BD) has been described as an alternative method to achieve internal biliary drainage in those patients in whom ERCP is not feasible. EUS-guided cholangiopancreatography (ESCP) was first described by Wiersema *et al*[96] in 1996. ESCP using either direct access or a rendezvous technique has shown a technical success between 75%-100%[97-100], although complications can reach up to 20%, especially in the early phase of the learning curve of the procedure[101].

ESCP can be performed through different routes (transgastric, transduodenal) and with different techniques (rendez-vous, hepaticogastrostomy, choledocoduodenostomy)[102]. In the rendezvous technique, the bile duct is punctured with a 19 or 22 G needle under EUS guidance and a wire is antegradely guided through any stricture and across the papilla under fluoroscopic guidance. The echoendoscope is then removed, leaving the wire in place, and the procedure is completed with a duodenoscope.

In hepaticogastrostomy and choledocoduodenostomy, the bile duct is punctured, preferably with a 19 G needle, a wire is guided into the bile duct and, after dilation of the transmural tract, a plastic or metallic stent is inserted.

EUS-GUIDED RADIOFREQUENCY ABLATION

EUS-guided radiofrequency ablation (EUS-RFA) has been successfully tested in two porcine studies for ablation of both lymph nodes[103] and the pancreas [104]. RFA was performed with a EUS adapted probe which was inserted through the lumen of a FNA needle. At histological analysis, the ablation effect was limited to the lesions and a direct correlation was seen between probe length and length and diameter of the necrosis.

EUS-FNI FOR TUMOR ABLATION AND INTRATUMORAL DRUG DELIVERY

EUS-FNI has made the intratumoral delivery of ethanol, chemotherapy as paclitaxel[105] or biological agents[106] possible in a precise real time tumor visualisation. Several studies have proved that it is a promising and safe technique, but validation in larger studies over longer follow-up periods is necessary.

EUS GUIDED RADIATION THERAPY

In a recent study with 22 patients with pancreatic cancer in which an average of 10 radioactive iodine-125 seeds were implanted under EUS guidance, the authors noticed a decrease in pain during the week following brachytherapy but there was no long-term survival benefit [107]. Recent reports concluded that EUS is safe for fiducial placement in pancreatic tumors [107] and for submucosal injection of tantalum for identification of the tumor during radiation and surgery[108].

In conclusion, EUS plays an important role in the diagnosis of pancreatic cancer, including FNA with cytological or histological confirmation. Staging of pancreatic cancer is crucial and CT and EUS are the cornerstones of staging, currently providing the more accurate results. Furthermore, EUS also has a therapeutic role, providing biliary drainage when it is not feasible with ERCP and pain relief. EUS can also have future applications on pancreatic cancer management.

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TOPIC HIGHLIGHT

Jose Manuel Ramia, MD, PhD, FACS, *Series Editor*

Reconstruction after pancreatoduodenectomy: Pancreatojejunostomy *vs* pancreatogastrostomy

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Abstract

Pancreatic surgeons try to find the best technique for reconstruction after pancreatoduodenectomy (PD) in order to decrease postoperative complications, mainly pancreatic fistulas (PF). In this work, we compare the two most frequent techniques of reconstruction after PD, pancreatojejunostomy (PJ) and pancreatogastrostomy (PG), in order to determine which of the two is better. A systematic review of the literature was performed, including major meta-analysis articles, clinical randomized trials, systematic reviews, and retrospective studies. A total of 64 articles were finally included. PJ and PG are usually responsible for most of the postoperative morbidity, mainly due to the onset of PF, being considered a major trigger of life-threatening complications such as intra-abdominal abscess and hemorrhagia. The included systematic reviews reported a significant difference only in the incidence of intraabdominal collections favouring PG. PF, delayed gastric emptying and mortality were not different. Although there was heterogeneity between these studies, all were conducted in specialized centers by highly experienced surgeons, and the surgical care was likely to be similar for all the studies. The disadvantages of PG include an increased incidence of delayed gastric emptying and of main pancreatic duct obstruction due to overgrowth by the gastric mucosa. Exocrine function appears to be worse after PG than after PJ, resulting in severe atrophic changes in the remnant pancreas. Depending on the type of PJ or PG used, the PF rate and other complications can also be different. The best method to deal with the pancreatic stump after PD remains questionable. The choice of method of pancreatic anastomosis could be based on individual experience and on the surgeon's preference and adherence to basic principles such as good exposure and visualization. In conclusion, up to now none of the techniques can be considered superior or be recommended as standard for reconstruction after PD.

Key words: Pancreatoduodenectomy; Pancreatojejunostomy; Pancreatogastrostomy; Pancreatic fistula; Pancreatic cancer; Surgical technique

Core tip: Pancreatoduodenectomy is a technique with a high rate of morbidity and mortality. Surgeons try to find the best technique of reconstruction in order to decrease postoperative complications. We compare the two most frequent techniques of reconstruction after pancreatoduodenectomy, namely pancreatojejunostomy and pancreatogastrostomy, to determine which of the two is better. We offer a systematic review of the main papers published with all the pros and cons of each technique. The best method to deal with the pancreatic stump after pancreatoduodenectomy remains questionable. The choice of method of pancreatic anastomosis could be based on individual experience and on the surgeon's preference and adherence to basic principles, such as good exposure and visualization.

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INTRODUCTION

Pancreatic surgeons try to find the best technique of reconstruction after pancreatoduodenectomy (PD) in order to decrease the frequency and seriousness of postoperative complications, mainly pancreatic fistulas (PF)[1].

The aim of this work was to compare the two most frequent techniques of reconstruction after PD, pancreatojejunostomy (PJ) and pancreatogastrostomy (PG) in order to determine which of the two is better.

PANCREATOJEJUNOSTOMY

Whipple's technique was described in 1935 and initially involved a two-time excision, performing bypass pathways before resection of the surgical specimen[2]. This name is reserved today to the resection of the pancreatic head and accompanying biliodigestive structures: gastric antrum, duodenal frame, first jejunal loop, gallbladder in continuity with the cystic duct and distal common bile duct.

After excision, reconstruction is needed. There are several ways but the best known is described by Child in 1943[3], consisting of successive drainage of the pancreas, bile duct and stomach in the first jejunal loop and still prevails today. This circuit is simple and ensures a rapid mixture of bile and pancreatic secretions.

More generally, to prevent backflow of one anastomosis to another, this type of reconstruction must follow these rules: (1) PJ is proximal to hepaticojejunostomy, which is proximal to gastrojejunostomy; (2) the distance between each small bowel anastomosis is ideally of at least 30-40 cm to limit food reflux into the biliary and pancreatic anastomosis; and (3) the anastomosis must be isoperistaltic. The first jejunal loop is usually mobile enough to place it in the supramesocolic compartment and allow these three anastomoses.

PJ is usually responsible for most of the postoperative morbidity[4], which currently remains high[5,6], mainly due to the onset of PF, being considered a major trigger of life-threatening complications such as intra-abdominal abscess and hemorrhagia[7]. Because of this, we have described several types of anastomoses, all aimed to reduce the rate of occurrence of the feared fistula.

Types of pancreatojejunostomies

Reconstruction methods between the pancreas and the small remnant include various forms ranging from end-to-side anastomosis, termino-terminal anastomosis or pancreatic intussusception in the jejunum. Of these, the most used are the invagination and duct-to-mucosa anastomosis without stenting the main pancreatic duct (MPD).

End-to-side PJ: This anastomosis has two variants which are: (1) direct anastomosis in a single plane, consisting of an anastomosis in a single plane between the upper and lower pancreatic edges and the longitudinal gap (3-4 cm) in the jejunum; and (2) duct-to-mucosa anastomosis, which is the most frequently used. The jejunal loop is placed with the fornix on the left, and in a slight clockwise rotation so that its antimesenteric edge is in contact with the pancreatic sectional area. A seromuscular longitudinal incision must be performed with length equal to the sectional area of the pancreas. The jejunal mucosa is incised on a limited basis against the MPD. The backplane of the seromuscular end-to-side anastomosis begins with a continuous suture from outside to inside in the pancreas (avoiding excessive pressure to prevent tearing) and then from inside to outside in the jejunum. The suture is started at the upper edge of the sectional area, ending in the lower part. Then, interrupted suture is performed taking the MPD wall and the mucosa of the jejunum to face the MPD. The anterior plan begins with a continuous suture that follows the same principles as the backplane. This suture is completed with a second angled stitch.

Both techniques were studied in a prospective randomized trial by Bassi *et al*[8] in which it was concluded that the rate of PF was lower after duct-to-mucosa anastomosis.

PJ by invagination: This anastomosis is acceptable when the remaining pancreas is thin and can enter the jejunum[9-11]. The principle of intussusception is to coat the entire bed of the pancreatic section with the wall of the jejunum to suppress PF that may come from the secondary conduits sectioned on the periphery of the bed or are exposed by a parenchymal necrosis due to the sutures which pass through the capsule.

There are three types of PJ by invagination: (1) classic end-to-end anastomosis. It is an end-to-end PJ performed with "U" stitches. Next, the pancreas is inserted into the jejunum and tied. This technique has not been evaluated in a randomized clinical trial; and (2) end-to-end anastomosis with invagination by Peng *et al*[12] (binding). Described by Peng *et al*[12] in 2002, the technique includes three modifications: (1) the jejunum is everted on itself to make a first anastomosis between the jejunal mucosa and the pancreas; (2) to improve cohesion between the pancreas and jejunum, the jejunal mucosa covering the pancreas along 2-3 cm is initially destroyed by chemical or thermal means to create an adhesion zone; and (3) a ligature is applied around the covered area after the procedure when the jejunum is properly arranged on the pancreas.

The results of this anastomosis were excellent in a randomized clinical trial conducted by the promoter of this technique[13], but so far have not been confirmed in two prospective studies in 2010[14,15]. More recently, a prospective, but not randomized, study showed that the

method described by Peng is safe but is not associated with a lower frequency of PF, morbidity or mortality in comparison with the duct-to-mucosa anastomosis[16]. End-to-side anastomosis with invagination by Grobmyer *et al*[17]. This anastomosis consisted of making a muco-mucosa anastomosis by a jejunal incision in the antimesenteric border of the small intestine and whose size is equivalent to the MPD, associating an invagination of the pancreatic bed in the seromuscular layer of the jejunum. For this, the side walls of the jejunum are fixed to the pancreatic capsule in order to cover the bed section.

This anastomosis has been successful in two comparable, retrospective series[17,18] and in a clinical randomized trial[19].

Comparing both types of anastomosis, duct-to-mucosa and invagination, the duct-to-mucosa anastomosis was initially described as safer and with a significantly lower rate of fistula[20,21]. Subsequently, in 2003 a prospective randomized trial[8] found PF in 14% of patients: 13% in the group with duct-to-mucosa anastomosis and 15% in the group with anastomosis by invagination, although the difference was not significant. A randomized prospective study in 2009[19] concluded that the invagination method significantly decreased the rate of PF *vs* duct-to-mucosa anastomosis (12% *vs* 24%, $P = 0.04$) in the pancreas with both soft and hard texture.

Anastomotic variants

Several alternatives to the above techniques have been described, all aiming to reduce the occurrence of a fistula and its consequences: (1) PJ with stent. The principle of stenting anastomosis is to derive the flow of pancreatic secretions with the aid of a catheter inserted in the MPD. We distinguish between lost drainage and externalized drainage (or internal-external drainage): Anastomosis with internal drainage consists of introducing a catheter with a diameter equivalent to the MPD during the anastomosis. Then the catheter migrates spontaneously (in a few days or weeks) to the jejunum and is evacuated by natural means. The effectiveness of this procedure has only been evaluated in a single randomized clinical and was negative[22]. This procedure seems especially useful to prevent stenosis of the pancreatic duct during anastomosis. Anastomosis with external drainage consists of introducing a catheter in the MPD then externalizing it through the intestinal wall (covering it or not according to Witzel's technique) and then through the abdominal wall. The drain is left without pinching for the first postoperative days (usually 10-14 d), then can be clamped once healing is achieved, so that the pancreatic secretion passes. It is removed 4-6 wk after surgery. Comparing the presence of external or internal drainage, a study by Tani *et al*[23] in 2010 concluded that there was no significant difference between the implementation of internal or external drainage, and concurred with a meta-analysis in which it was stated that internal drainage does not affect the development of fistulas and is not useful in a soft pancreas[24]. Comparing the use of external drainage or use of none, there is a study which states that the range of PF between external drainage or no drainage is similar, with no decrease in the rate (11.5% *vs* 14.8%; $P = 0.725$) with the use of external drainage[25]. A meta-analysis of randomized controlled trials most recently by Hong *et al*[26] concluded that the application of external drainage after pancreatoduodenectomy can decrease the incidence of pancreatic leakage compared with the use of any drainage. This technique is discussed in a different section.

PANCREATOGASTROSTOMY

PJ, and variations thereof, has been the technique most frequently used, although PG is a good alternative. In 1934, Tripodi performed a PG in a dog, and reported adequate pancreatic secretion postoperatively[27]. The first PG in humans was performed in 1944[28]. Since then, several series with around 3800 patients have been published[29-31], and their outcomes have been compared in some papers with those of PJ to determine the best reconstructive technique.

Types of pancreatogastrostomies

Basically, three types of PG have been described: (1) in classic duct-to-mucosa anastomosis the pancreatic stump is sutured to the seromuscular layer of the gastric wall, while the MPD is sutured to the full-thickness stomach[32], with or without a lost pancreatic stent; (2) in pancreatic stump intussusception into the stomach, the distance between the surface of the stump and the suture is longer, thus decreasing the risk of a fistula between the stitches that cross the pancreatic capsule. Suturing can be performed from the posterior gastric surface or from the inside of the gastric cavity through an anterior gastrostomy[33]. Transverse gastrotomy seems to be associated with a higher incidence of delayed gastric emptying[34,35] compared with a longitudinal incision[32,36]; and (3) in the exteriorized pancreatic stent, the tube introduced into the pancreatic duct passes through the anterior gastric wall and the abdominal wall. Drainage may be closed 10–14 d later and removed 4-6 wk after surgery.

Alternative procedures include a binding or purse string suture around the anastomosis in the gastric wall[37], with complete stitches traversing the anterior and posterior surface of the pancreatic stump associated with a duct-to-mucosa anastomosis[38] or a "gastric partition" where the PG is performed[39]. An aspirating nasogastric tube is always recommended. At any rate, there are no studies showing the superiority of any of these techniques.

Definition of pancreatic fistula

The most frequent complications after PD are delayed gastric emptying, PF, postoperative bleeding and intra-abdominal abscess[40-43]. Although mortality has dramatically decreased from higher than 20% in the 1980s to less than 5% nowadays[40,44-47], morbidity remains around 40%-50%[48,49]. Differences in the definitions of these complications have led to a consensus of the International Study Group for Pancreatic Surgery (ISGPS) in 2006.

PF appears in 3%-30% of patients[1,41,50,51]. It must be suspected when the amylase content of drained fluid is more than 3 times the normal value in the third postoperative day. ISGPS classifies fistulas as: (1) grade A (patient is stable, has a transient fistula and no collections in computed tomography); (2) grade B (patient needs parenteral nutrition, antibiotics and somatostatin and has peripancreatic collections that can be percutaneously drained); and (3) grade C (patient needs to be under intensive care, have percutaneous drainage of the collections or surgery to repair the leakage, to change from PJ to PG or to do a total pancreatectomy)[43,52].

Advantages of pancreatogastrostomy over pancreatojejunostomy

The technique of PG has several potential advantages over PJ. It can be performed easily, because the posterior wall of the stomach lies immediately anterior to the mobilized pancreatic remnant and is usually wider than the transected pancreas. The posterior wall of the stomach is thick and highly vascularized compared with the jejunum. PG anastomosis is then located at a certain distance away from the major blood vessels, which are skeletonized during the resection phase of the tumor and the lymph nodes. If a PF occurs after PG, the major vessels are less prone to being damaged by activated proteolytic enzymes of the pancreas[53].

In PG, the pancreatic exocrine secretions enter the potentially acidic gastric environment, precluding digestive damage of the pancreatoenteric anastomosis by activated proteolytic enzymes. In PJ, the activation of pancreatic exocrine secretions can occur more easily in the presence of intestinal enterokinase and bile. These factors can easily cause digestive damage to the anastomosis and the major vessels in the presence of abundant proteolytic enzymes escaping from the fistula[35].

PG avoids the long jejunal loop where pancreatobiliary secretions accumulate during the early postoperative period and reduces the number of anastomoses in a single loop of retained jejunum, which potentially decreases the likelihood of loop kinking[53]. Postoperative gastric decompression can result in removal of gastric and pancreatic secretions. It also avoids tension on the anastomosis. A nasogastric tube can be used as drainage if a fistula occurs after PG, thereby avoiding potentially invasive procedures[53].

The decreased morbidity of intra-abdominal complications for PG may be the result of the aforementioned theoretical advantages.

Comparison of both techniques

To compare both techniques of reconstruction, five randomized trials[39,52,54-56] and several meta-analysis and systematic reviews[16,22,53,57-64] have been published in the recent years. Systematic reviews included 553 patients and found a significant difference only in the incidence of intraabdominal collections favoring PG (OR = 0.46; 95%CI: 0.26-0.79; P = 0.005). PF, delayed gastric emptying and mortality were not different. The recent paper by Topal *et al*[56] included 329 patients and showed a lower incidence of PF after PG (OR = 2.86; 95%CI: 1.38-6.17; P = 0.02). Although there was heterogeneity between these studies, all were conducted in specialized centers by highly experienced surgeons and the surgical care was likely to be similar for all the studies.

It is generally accepted that, compared with a fibrotic pancreatic remnant, a soft and fragile pancreatic stump frequently results in a high rate of pancreatic anastomosis leakage[59]. Among the conditions which can lead to PF, pancreatic texture, pancreatic stump blood supply, pancreatic duct size and pancreatic juice output are important factors[43,52].

Disadvantages of PG have been identified, including an increased incidence of delayed gastric emptying and of MPD obstruction due to overgrowth by the gastric mucosa. Available data on hormone levels indicate that the exocrine function appears to be worse after PG than after PJ, resulting in severe atrophic changes in the remnant pancreas[60].

Other factors such as presenting symptoms, preoperative blood parameters, the presence of comorbid illness and preoperative biliary drainage that may influence the frequency or type of morbidity, were not usually considered. Furthermore, the definition of PF also varied between these articles, with only two studies[39,56] applying the ISGPF criteria. Also, none of the papers considered stratification of the patients by MPD diameter, which also seems to correlate strongly with pancreatic texture[53].

The reported technique for PD was variable. From the article published by Fernández-Cruz *et al*[39], with 100% of patients having a pylorus-preserving modification (PPPD) and no patient with the classic Whipple procedure, to that by Topal *et al*[56], with 61% and 39% of patients having the respective procedures. There were also variations of the PJ technique that could be associated with differences in the PF rate. Three randomized trials show a lack of uniform technique[52,54,55]. A duct-to-mucosa technique was used as the standard in one trial[52] and at the surgeon's discretion in another two trials[55,56]; end-to-end PJ was used in two trials at the surgeon's discretion[54,55]; and a duct-to-mucosa PJ with an internal stent was used in only one trial[39].

The techniques of PG were also different in the five randomized trials[39,52,54-56]. In one paper[54], the pancreatic anastomosis used the classical technique first described, two randomized trials used the second technique[52,56] and in another trial the details of PG anastomosis were not mentioned[55]. The lack of a uniform technique for PG raises the same controversy as for PJ, since different operative procedures could reasonably lead to different complications.

A new technique, PPPD with gastric partition was described only in the study by Fernández-Cruz *et al*[39]. Although this technique was associated with lower rates of postoperative fistula than PJ, this surgical technique is not easy to reproduce and might not always be possible for oncological reasons[56]. This complexity may explain why gastric partitioning with preservation of the pylorus and

the gastro-epiploic arcade, together with the placement of a pancreatic stent through the anastomosis, is still not implemented in most centers.

Ways to decrease complications

Use of occlusive substances: Neoprene injection[61] in the MPD to occlude the duct thus neutralizing exocrine pancreatic secretion is an option that has not reduced the rate of PF according to a randomized clinical trial[62]. Another recent randomized trial evaluated the effect of topical fibrin glue applied externally to all anastomoses after PD. The conclusions of this study are that fibrin glue application does not reduce the incidence of anastomotic leaks[62].

Use of somatostatin: Somatostatin and somatostatin analogues (octreotide) was used in all patients in the studies by Bassi *et al*[52], Topal *et al*[56] and at the surgeon's discretion in the study by Duffas *et al*[55]. However, somatostatin was not used prophylactically in any patients in the studies by Yeo *et al*[54] and Fernández-Cruz *et al*[39]. Prophylactic use of somatostatin and octreotide in pancreatic surgery remains controversial and several meta-analyses came to contradictory conclusions. A more recent meta-analysis of randomized trials on the effectiveness of somatostatin analogues for pancreatic surgery[63] concluded that somatostatin analogues reduce postoperative complications but do not reduce perioperative mortality, and they do shorten hospital stay in patients undergoing pancreatic surgery for malignancy. For this reason, adequately powered trials with a low risk of bias are necessary.

Although some long-term outcomes show that exocrine function after PG is decreased compared with PJ, available data on hormone levels indicate that endocrine function appears to be similar. Despite these results, the benefits resulting from a reduction in occurrence of postoperative PF are higher[35].

Wrapping: Use of the omentum or falciform ligament to wrap local retroperitoneal vessels in pancreaticojejunal anastomosis. Its use in the West is limited. It is used for two purposes: (1) to avoid the autolytic effect and proteolytic activity of pancreatic juice and infected fluids on surrounding organs, especially the abdominal vessels. This is intended to reduce the postoperative bleeding rate; and (2) to reduce the rate of PF by avoiding complications arising from it.

Wrapping is not exempt from complications such as panniculitis, intestinal obstruction, necrosis of the omentum, and intrabadominal abscess. In some patients over or under size, it cannot be used.

The falciform ligament shares a percentage of the features we have discussed for the omentum, but it is smaller and shorter so it can be used to cover vascular structures but it is hard to wrap a PJ. A great advantage is that no complications have been associated with its use.

The literature on wrapping in oncologic pancreatic surgery is rare, and usually consists of retrospective studies with a low level of evidence,and studies mixing different types of pancreatic surgery and various wrapping techniques. It seems that wrapping slightly decreases postoperative bleeding and PF, and when this occurs is less severe than when not using wrapping. However, a prospective randomized trial is needed to let us know if we can use the technique more generally[64].

Use of stents

Only in one randomized trial are stents used[39]. The benefit of an internal or external stent across pancreaticoenteric anastomosis remains controversial. Two prospective randomized trials have reached different conclusions on the benefit of stenting in reducing the PF rate[22,50]. Winter *et al*[22] found that the use of a short internal stent did not reduce the frequency or the severity of pancreatic fistula after PJ. In their study the technique of PJ anastomosis was not standardized. Poon *et al*[50] used an end-to-side, duct-to-mucosa anastomosis, and the patients were randomized to have either an external stent inserted across the anastomosis to drain the pancreatic duct or no stent. This trial showed a reduction in the incidence of PF from 20% in the non-stented group to 6.7% in the stented group.

CONCLUSION

The best method to deal with the pancreatic stump after PD remains in question even. The choice of method of pancreatic anastomosis could be based on individual experience and on the surgeon's preference and adherence to basic principles such as good exposure and visualization. It is important to suture placement without choking the MPD to not produce a watertight anastomosis and preservation of the blood supply. In conclusion, up to now none of the techniques can be considered as superior and recommended as standard for reconstruction after PD. Future large-scale, high-quality, multicenter trials are required to clarify the issues of reconstruction following PD.

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CASE REPORT

Colonic adenocarcinoma, mucosa associated lymphoid tissue lymphoma and tuberculosis in a segment of colon: A case report

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Abstract

Synchronous occurrence of adenocarcinoma and mucosa associated lymphoid tissue (MALT) lymphoma of colon is rare, and its presence with coexisting tuberculosis is still rarer. To our knowledge, this may be the first case report. In the present report, we describe a 43-year-old female who presented with a history of abdominal pain, fever, loss of weight and loss of appetite. Colonoscopy showed a large ulceroproliferative mass arising from the caecum, biopsy of which showed it to be adenocarcinoma of the colon. A right hemicolectomy was performed and microscopic study of the colon revealed tuberculosis and synchronous adenocarcinoma with lymphoma. Eight of sixteen lymph nodes showed tuberculosis and three of sixteenpericoloniclymphnodes showed metastatic deposits. Immunostains further confirmed the tumour to be adenocarcinoma with MALT lymphoma. We would like to highlight the diagnostic challenges arising from the multi-faceted presentations of these three conditions.

Key words: Adenocarcinoma;Mucosa associated lymphoid tissue lymphoma; Tuberculosis

Core tip: We report a first case report of synchronous adenocarcinoma,mucosa associated lymphoid tissue (MALT) lymphoma and tuberculosis in the same segment of colon in 43-year-old immunocompetent female patient. There are around 4 case reports of sychronous adenocarcinoma and MALT lymphoma to date in the literature.What we describe isthe first such case in the literature.

Kulandai Velu AR, Srinivasamurthy BC, Nagarajan K, Sinduja I. Colonic adenocarcinoma, mucosa associated lymphoid tissue lymphoma and tuberculosis in a segment of colon: A case report. *World J Gastrointest Oncol* 2014; 6(9): 377-380 Available from: URL: http://www.wjgnet.com/1948-5204/full/v6/i9/377.htm DOI: http://dx.doi.org/10.4251/wjgo.v6.i9.377

INTRODUCTION

Mucosa-associated lymphoid tissue (MALT) tumors are a distinct subtype of non-Hodgkin's lymphoma associated with predisposing infectious or autoimmune processes, resulting in chronic lymphoid proliferation. Though the stomach is the most common site, MALT tumor has been reported in non-gastric sites like salivary gland, lung, ocular adnexa and skin[1]. The colon is a rare location for MALT lymphoma[2]. Synchronous colonic adenocarcinoma and malignant lymphoma in the same patient is rare with an estimated incidence of around 0.0002%[3]. Only a few cases have been reported in literature.Adenocarcino-

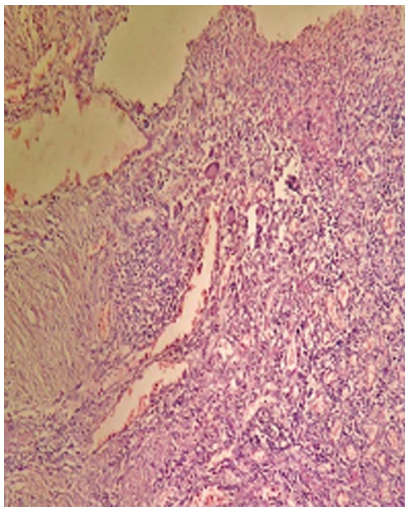

Figure 1 Histology showed extensive mucosal necrosis surrounded by lymphocytes and langhans giant cells.

ma and tuberculosis occurring at the same site is exceedingly rare. Chronic inflammatory mucosal damage initiating a sequence of metaplasia and dysplasia could result in neoplastic changes[4]. We describe a case report never reported in literature before, sychronous adenocarcinoma and lymphoma with tuberculosis of the colon which poses a diagnostic and therapeutic challengeespecially when the patient can present with equivocal symptoms.

CASE REPORT

A 43-year-old female was referred with a history of abdominal pain, fever, loss of weight and loss of appetite for 6 mo. Hematological investigations showed normocytic normochromic anemia with a raised erythrocyte sedimentation rate. Chest roentgenogram was normal. Human immunodeficiency virus antibodies were negative. Colonoscopy revealed an ulcero-proliferative mass arising from the caecum. Ultrasonography revealed a thickened caecal wall with mesenteric lymphadenopathy. A biopsy diagnosed it as adenocarcinoma. A right hemicolectomy was performed. The gross pathological examination of the lesion showed a 4 cm × 3.5 cm × 3 cm ulcero-proliferative tumour present on the mucosal surface. The entire mucosal surface appeared normal without any abnormality or polypoidal lesion. Sixteen pericolic lymph nodes varying in size from 0.5 to 3 cm were isolated from pericolic fat. Sections from ulcero-proliferative growthrevealed extensive mucosal necrosis with ill defined granuloma, langhans giant cells (Figure 1) and moderately differentiatedadenocarcinomathat extended through the muscularis propria into the subserosal adipose tissue (Figure 2A). Dense lymphocyticinfiltration was seen in the submucosa. These lymphoid cells were small to medium sized cells with mildly irregular nuclear contours anda moderate pale cytoplasm (Figure 2B). Thus, microscopic study revealed tuberculosis with tumour and the tumour type to be synchronous adenocarcinoma with lymphoma. The adenocarcinoma component was moderately differentiated while the lymphoma component was of low grade MALT lymphoma.The surgical cut margins were free of tumor. Eight of sixteen lymph nodes showed features of tuberculosis with acid fast bacilli in two of the lymph nodes and three of sixteen pericoloniclymphnodes showed metastatic deposits (Figure 3B). A tissue section from mucosa did not reveal acid fast bacilli.Immunohistochemical analysis was performed on representative sections from the colon and lymph node to characterize the lymphoid cells and to confirm adenocarcinoma.CD 20 (Dako preparation) was diffusely positive (Figure 4A) and CD5 was negative in neoplastic lymphocytes. Cytokeratin and epithelial membrane antigen (Figure 3A and Figure 4B) was positive in sections from colon, pericolonic-tumor and metastatic deposits in lymphnodes.

After surgery, the patient was put on anti-tubercular treatment. No other adjuvant therapy was started as the patient was not willing; the patient is alive and well after 6 mo post-operatively.

DISCUSSION

Our case, to the best of our knowledge, is the first ever reported case of synchronous adenocarcinoma and lymphoma with tuberculosis. An association of TB and malignancy has been noted by several authors in different organs[4]. Some authors have proposed that the association of carcinoma and tuberculosis is coincidental[5]. In our case both occurred at the same segment of colon and it is justified to think that the inflammatory condition has facilitated malignancy and the impaired immune mechanism has further facilitated the development of second malignancy. In 1987, Tanaka *et al*[6] analysed 26 TB and adenocarcinoma cases reported in Japan and supported the possibility of cancer originating from a tuberculous lesion. Chronic inflammatory mucosal damage initiating a sequence of metaplasia and dysplasia may result in neoplastic change. On the other hand an impaired host immune response due to malignancy would have reactivated the dormant tubercular lesion. However, it is still a matter of debate and further research is required to determine if a tuberculous infection, being similar to other chronic infections and inflammatory conditions, may facilitate carcinogenesis or the malignancy which reactivates the infection[7]. Devi *et al*[8] and Argyropoulos *et al*[9] first reported a case of synchronous adenocarcinoma and MALT lymphoma in athesame segment of colon followed by a series of three cases by Argyropoulos in 2012. Occurrence of secondary MALT-type lymphoma in a patient with prior colon adenocarcinoma after colectomy has been reported in the literature[10]. It is extremely difficult to diagnose synchronous tumours in the same segment.In our case, a dense lymphocytic infiltratenoted in the vicinity of adenocarcinomaalerted us to thoroughly sample the specimen and to assess the immunophenotype by immunohistochemistry which was of great help

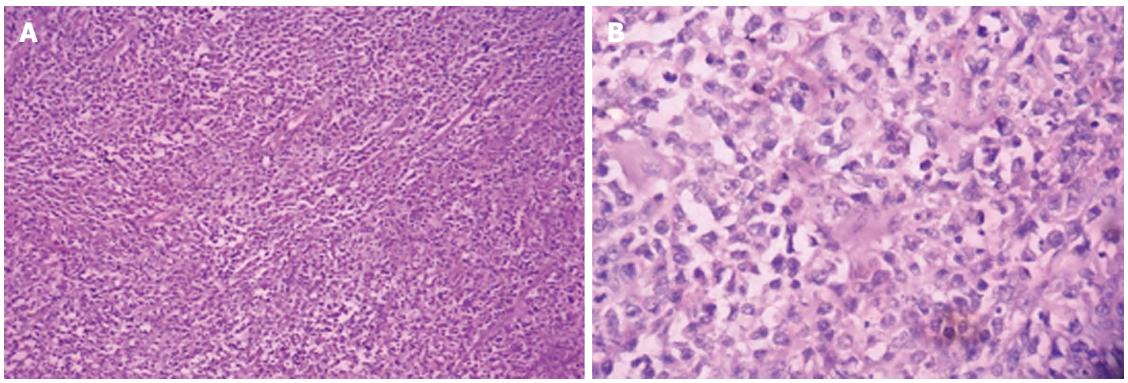

Figure 2 Histology showed moderately differentiated adenocarcinoma infiltrating the submucosa and serosa (A: HE, × 10) with mitotic figures and surrounded by neoplastic lymphocytes (B: HE, × 40).

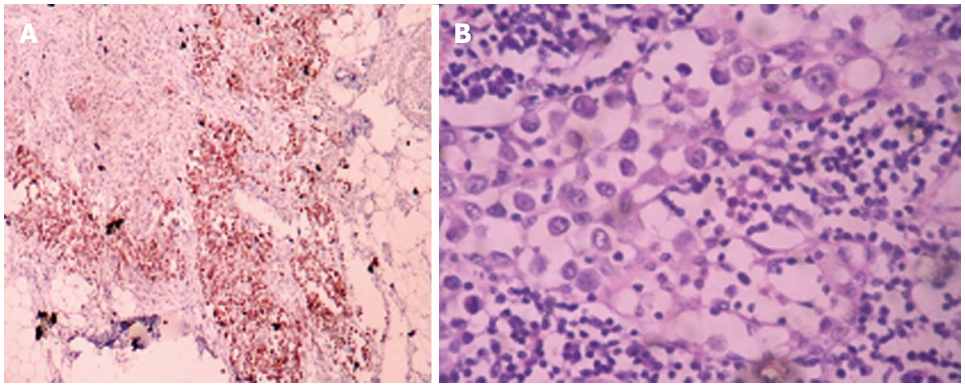

Figure 3 Pericolonic fat infiltrated by adenocarcinoma showing cytokeratin positivity (A: IHC, × 10), histology of lymph node showing metastatic deposits of adenocarcinoma (B: HE, × 40).

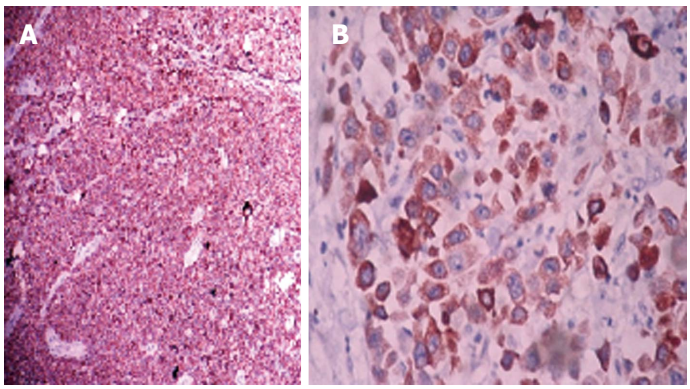

Figure 4 Mucosa associated lymphoid tissue lymphoma showing CD20 positivity(A: IHC, × 4) and diffuse cytoplasmic positivity of cytokeratin in pericolonic tissue suggestive of adenocarcinoma(B: IHC, × 40).

in confirming the diagnosis.

COMMENTS

Case characteristics

A 43-year-old female referred with history of abdominal pain, fever, loss of weight and loss of appetite for 6 mo.

Clinical diagnosis

Colonoscopy revealed ulcero-proliferative mass arising from the caecum.

Differential diagnosis

Tuberculosis, adenocarcinoma of the colon.

Laboratory diagnosis

Normocytic normochromic anaemia, raised erythrocyte sedimentation rate, human immunodeficiency virus antibodies negative.

Imaging diagnosis

Ultrasonography revealed thickened caecal wall and Mesenteric lymphadenopathy.

Pathological diagnosis

Synchronous adenocarcinoma, mucosa associated lymphoid tissue lymphoma and tuberculosis of a segment of colon.

Treatment

Right hemicolectomy.

Related reports

On immunohistochemical stain, CD20, cytokeratin and epithelial membrane antigen were positive.

Experiences and lessons

Impaired host immune response due to malignancy can reactivate the dormant tubercular lesion. It is extremely difficult to diagnose synchronous tumours in the same segment. In this case, a dense lymphocytic infiltrate noted in the vicinity of adenocarcinomaalerted the authors to thoroughly sample the specimen

and to assess the immunophenotype by immunohistochemistry which was of great help in confirming the diagnosis.

Peer review

This paper is the first report of synchronous adenocarcinoma and lymphoma with tuberculosis. This is an interesting case report.

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AIM AND SCOPE

World Journal of Gastrointestinal Oncology (*World J Gastrointest Oncol*, *WJGO*, online ISSN 1948-5204, DOI: 10.4251) is a peer-reviewed open access academic journal that aims to guide clinical practice and improve diagnostic and therapeutic skills of clinicians.

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REVIEW

Metastatic tumors to the pancreas: The role of surgery

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Abstract

Pancreatic metastases from other primary malignancies are a rare entity. By far, the most common primary cancer site resulting in an isolated pancreatic metastasis is the kidney, followed by colorectal cancer, melanoma, breast cancer, lung carcinoma and sarcoma. Only few data on the surgical outcome of pancreatic resections performed for metastases from other primary tumor have been published, and there are no guidelines to address the surgical treatment for these patients. In this study, we performed a review of the published literature, focusing on the early and long-term results of surgery for the most frequent primary tumors metastasizing to the pancreas. Results for the Literature's analysis show that in last years an increasing number of surgical resections have been performed in selected patients with limited pancreatic disease. Pancreatic resection for metastatic disease can be performed with acceptable mortality and morbidity rates. The usefulness of pancreatic resection is mainly linked to the biology of the primary tumor metastasizing to the pancreas. The benefit of metastasectomy in terms of patient survival has been observed for metastases from renal cell cancer, while for other primary tumors, such as lung and breast cancers, the role of surgery is mainly palliative.

Key words: Pancreas; Pancreatic neoplasms/secondary; Pancreatectomy; Renal cell cancer; Breast cancer; Melanoma; Sarcoma; Lung carcinoma

Core tip: Pancreatic metastases represent a rare but increasing entity among pancreatic tumors. We have reviewed the literature's reports of the more common metastatic tumors to the pancreas, evaluating early and long-term results of surgery. Pancreatic resection may appear a safe and feasible option also in metastatic tumors, but long term survival is achieved substantially only in renal cell cancer. In other metastatic tumors, pancreatectomy may offer a good palliation in selected patients, but it is to remark that surgery is only one option in the multimodality treatment of metastatic disease to the pancreas.

Sperti C, Moletta L, Patanè G. Metastatic tumors to the pancreas: The role of surgery. *World J Gastrointest Oncol* 2014; 6(10): 381-392 Available from: URL: http://www.wjgnet.com/1948-5204/full/v6/i10/381.htm DOI: http://dx.doi.org/10.4251/wjgo.v6.i10.381

INTRODUCTION

Pancreatic metastases from other primary cancers are rare[1]. Approximately 2% of pancreatic cancers are metastatic from other primary site[2,3]. In different autopsy series, a wide range of malignant tumors have been found to metastasize to the pancreas and the most frequent primary locations of tumor were the kidney, breast, colon, skin and lung[4-6]. It may be difficult to differentiate a pancreatic metastasis from a primary pancreatic tumor, being the clinical presentation and the radiological characteristics similar for both primary and secondary neoplasms[7,8]. Pancreatic metastases are asymptomatic in more than 50% of cases: they are often detected during follow-up

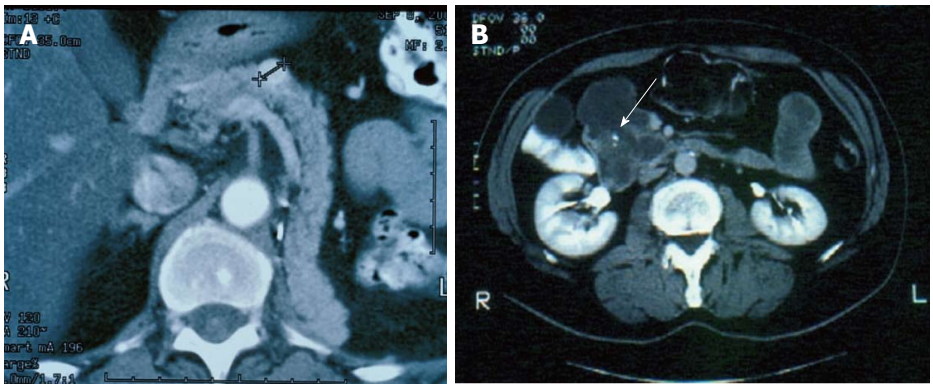

Figure 1 Computed tomography scan of the abdomen. A: Computed tomography (CT) scan of the abdomen showing a contrast-enhanced pancreatic metastasis from a renal cell carcinoma; B: CT scan of the abdomen showing an hypodense metastatic lesion of the pancreatic head from a colon carcinoma.

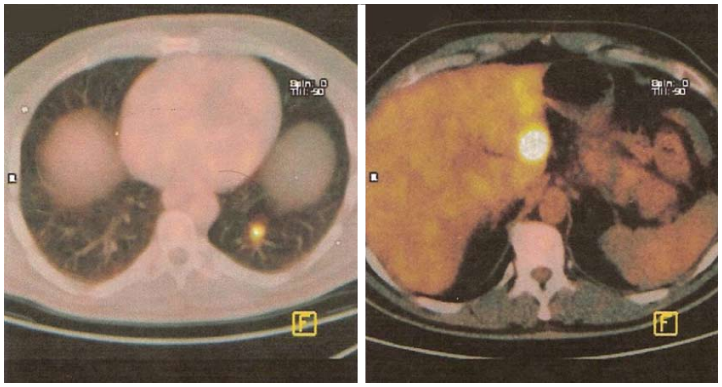

Figure 2 Positron emission tomography/computed tomography imaging showing a pathologic uptake of the tracer in the region of the pancreatic neck and in the left lung from a melanoma.

investigations after surgery for a primary lesion or as an incidental finding on imaging studies performed for an unrelated condition[9,10]. At CT scan, pancreatic metastases may appear as hypervascular lesions, like in renal cell cancer (RCC) metastases (Figure 1A) or, as in the case of colon and melanoma metastases, as hypodense masses (Figure 1B). Positron emission tomography may be helpful in order to exclude other metachronous lesions than the pancreatic one or other primary synchronous tumors (Figure 2).

Pancreatic metastases occur in two different clinicopathological settings, either as one manifestation in widespread disease or as an isolated mass of the pancreas. However, only few patients present with a single potentially resectable pancreatic lesion[11] and the most common presentation is that of a widespread metastatic disease[12]. The number of pancreatic resections for metastatic lesions in high volume centers has gradually increased, probably because of the greater knowledge of these clinical entities and the greater availability of radiological studies in asymptomatic patients[13]. In recent years, different studies showed an improved survival in patients undergoing lung or liver resection for metastatic lesions from colorectal cancer[14,15]. Pancreatic resections were for many years associated with high rates of morbidity and mortality, but recent data have clearly shown that pancreatic surgery is safe and feasible in high-volume clinical centers: the lower morbidity and mortality rates make pancreatic resection an acceptable indication also in case of metastatic lesions[16-18].

In this study, we have reviewed the literature's reports of the more common metastatic tumors to the pancreas, evaluating early and long-term results of surgery.

RESEARCH

The published Literature was systematically searched using PubMed and free text search engines up to October 2013. Search terms included: pancreatic neoplasms/secondary, pancreatectomy, renal cell cancer, breast cancer, melanoma, colorectal cancer, sarcoma, lung cell cancer. The "related articles" function was used to broaden the search and all abstracts, studies, and citations retrieved were reviewed. Only articles published in the English language, with abstracts, and human studies only were selected. Case reports were included for the less common neoplasms. In the case of sequential publications, the report with the most comprehensive information regarding the study population was selected. Studies were excluded from the analysis if: (1) the outcome and parameters of interest were not clearly reported, and (2) it was impossible to extract the data from the published results. Two investigators (LM and GP) reviewed the titles and abstracts and assessed the full text of the articles obtained to establish eligibility. The following data were extracted from each study: first Author, year of publication, number of patients, perioperative morbidity and mortality, and long-term outcome. For statistical analysis, overall

averages are presented as weighted means (range) unless otherwise stated.

The preliminary literature search showed 1536 studies matching the initial search criteria. After screening, 108 studies evaluating metastases to the pancreas were selected. There were 41 case series (more than two patients) and 67 single case reports, for a total of 418 patients with secondary tumor of the pancreas: metastases were mainly from RCC (n = 293), followed by melanoma (n = 38), colorectal cancer (n = 37), breast cancer (n = 19), sarcoma (n = 18), and lung cancer (n = 13).

The results of the Literature's review are showed for each tumor considered.

RCC

By far, the most common primary cancer site resulting in an isolated pancreatic metastasis is the kidney. RCC accounts for approximately 2% of all adult malignancies. Among kidney-limited diseases, RCC has a high overall survival rate (up to 95%)[19]. However 20% to 30% of patients have metastases at presentation, and the 5-year survival rate is less than 10% once metastases spread[20]. In autopsy series in primary RCC, pancreatic metastases were noted in 1.3% to 1.9%[21]. Hirota *et al*[22] revealed that a characteristic of the patients in this group was the long disease-free interval from the time of the nephrectomy to the diagnosis of metastatic disease. This long disease free interval indicates a biological pattern of slow growth, favouring local surgical resection. Pancreatic metastases are often the only metastatic lesions and they seems related to a good prognosis[17,23]. Pancreatic metastases are only rarely symptomatic; therefore a long follow-up (> 10 years) is indicated in patients with RCC[10]. At CT scans metastases from RCC appear as hypervascular lesions, and a differential diagnosis must be done with primary endocrine tumors[24]. OctreoScan® scintigraphy is not always able to differentiate neuroendocrine lesions from pancreatic metastases from RCC. A recent study on metastatic RCC showed the presence of positive scintigraphy, and thus the presence of somatostatin receptors, in 9 of 11 cases[25]. A percutaneous fine-needle biopsy to confirm the clinical suspicion is seldom necessary. Pancreatic metastases from RCC can occur a long time after the diagnosis of the primary RCC. The presence of synchronous pancreatic lesions is less frequent (15%-27% of cases)[22,26,27] and it may be an expression of a widespread disease, thus limiting the benefit of a pancreatic metastasectomy. In a recent review by Masetti *et al*[28], univariate analysis showed that a disease-free survival time less than 2 years in metachronous metastases was associated with a worse survival. The detection of multiple pancreatic metastases occurs more often in RCC than in other primary malignancies and this must be taken into account in the planning of the surgical treatment of these patients[23]. In a review of the literature we found 29 studies reporting on pancreatic resection for metastatic RCC (Table 1, [3,9,10,12,16,23,24,28-49]). Only reports with detailed clinical and follow-up informations on 2 or more patients were selected, while single case-reports were excluded. Informations on 293 patients have been published. Among these, the median interval between nephrectomy and pancreatic recurrence was 104 mo (range 0-348 mo). Perioperative mortality occurred in only 4 patients with a mortality rate of 1.5%. Morbidity was difficult to assess because this information wasn't always reported and because in many reports it wasn't possible to differentiate morbidity rate after resection for RCC from other primary tumors. Among the available data, the overall morbidity rate was 13.3%. Median follow-up was 36.8 mo (range 3-130 mo). Eighty patients died and among them 56 patients died of recurrent disease (in some reports this information was not available). Tanis *et al*[34], in a recent review of 421 patients undergoing resection of pancreatic RCC metastases, reported an actuarial 5 years survival rate, calculated on 321 patients for which data were available, of 72.6% and the survival of these patients was compared to that of 73 non-surgically treated patients: 2 and 5 years overall survival rates were 80% and 72% in the operated group and 41% and 14% in the non-operated group. Bassi *et al*[17] reported in a single-centre series a great 5-year survival benefit after surgical resection compared with conservative treatment of unresectable disease (53% *vs* 26%). Pancreatic metastases from RCC are reported to have a better prognosis when compared to other primary tumors, therefore an aggressive treatment, *i.e.*, surgical resection, should be considered in these patients. Reddy *et al*[9] demonstrated that the median survival for pancreatic metastases from RCC was 4.8 years *vs* 0.9 years for metastases from melanoma. Konstantinidis *et al*[12] reported a 5-year actuarial survival of 61%, and they demonstrated that RCC patients had a better median survival (8.7 years) compared to other pathologies. Chemotherapy, immunotherapy, and radiotherapy have generally proved to be ineffective for primary RCC or metastatic disease. Despite promising results with immunotherapy using IL-2, a complete response occurred in less than 15% and was rarely durable[50,51]. In more recent years several angiogenetic agents (bevacizumab, sunitinib, sorafenib) have showed promising results[52]. Therefore a multidisciplinary approach has to be recommended in the treatment of pancreatic metastates from RCC and further studies are needed to establish the way to combine surgery with medical treatment in the different periods of the disease.

Colorectal cancer

In the English Literature only few studies on pancreatic resection for metastatic colorectal cancers have been published so far[53], representing only single case reports, rarely more than two patients[54]. In recent years, several studies demonstrated encouraging results on surgical resections for metastatic colorectal cancer to the liver and lung; on the other hand, only few data are available for pancreatectomies in metastatic colorectal cancer[55]. In a review of the literature, we selected 24 studies regarding surgical treatment of pancreatic metastases from colorectal cancer (Table 2[9,24,26,29,37,54-72]). Informations on

Table 1 Pancreatic resections for metastates from renal cell carcinoma

Ref.	No. of patients	Treatment	Mortality-morbidity	Follow-up; (mo) median (range)	Dead
Niess *et al*[29]	16	DP (10); PPPD (3); PD (2); TP	0-NA	39 (4-76)	6 (37.5%)
Yazbek *et al*[30]	11	NA	5/11/2001	78 (12-108)	4 (36.3%)
Alzahrani *et al*[31]	12 (7 resected)	DP (3); TP (2); CP; PD	1/7/2000	19 (1-96)	5 (41.6%)
D'Ambra *et al*[32]	8 (7 resected)	NA	0–3/7	43 (12.9-74.5)	NA
You *et al*[33]	7	NA	0-NA	34 (7-69)	1 (14.3%)
Konstantinidis *et al*[12]	20	NA	0-NA	36.8 (0.5-143)	NA
Masetti *et al*[28]	6	TP (5); PD	1/6/2000	3	0
Tanis *et al*[34]	10	NA	0-NA	NA	3 (30%)
Zerbi *et al*[10]	36 (23 resected)	DP (11); enucleation (5); PD (4); TP (2); CP	0-14/23	31 (12-98)	9 (25%)
Reddy *et al*[9]	21	NA	0-NA	57.6 (4.2-219.6)	19 (90.5%)
Schauer *et al*[35]	10	TP (5); PD (3); PPPD; DP	2/10/2001	56 (56-60)	NA
Karimi *et al*[36]	3	DP (3)	NA	96 (60-156)	0
Eidt *et al*[37]	7	PPPD (4); TP (2); DP	0-NA	36 (12-156)	2 (28.6%)
Sellner *et al*[23]	3	NA	0–NA	48 (36-60)	0
Crippa *et al*[24]	5	DP (3); PPPD; PD	0-NA	41 (21-95)	1 (20.0%)
Wente *et al*[38]	15	DP (7); PD (3); TP (3); PP (2)	4/15/2000	10 (1-28)	1 (6.7%)
Jarufe *et al*[39]	7	NA	1-NA	24	NA
Moussa *et al*[40]	10 (7 resected)	PD (6); TP	1–NA	61	6 (60.0%)
Law *et al*[41]	14	NA	0–NA	130 (32-315)	3 (21.4%)
Sperti *et al*[16]	2	TP; CP + enucleation	0–NA	18 (14-21)	1 (50.0%)
Zacharoulis *et al*[42]	3 (2 resected)	NA	2/3/2000	26 (7-88)	0
Yachida *et al*[43]	5	NA	0–NA	12 (2-160)	0
Faure *et al*[44]	8	PD (5); TP (3)	1/8/2000	38 (13-83)	2 (25.0%)
Sohn *et al*[45]	10	PPPD (5); DP (2); PD (2); TP	0-3	8 (3-117)	2 (20.0%)
Ghavamian *et al*[46]	11	DP (8); TP (3)	0-NA	50 (5-120)	3 (27.3%)
Kassabian *et al*[47]	5	CP; PPPD; TP; PD; DP	0-NA	48	1 (20.0%)
Thompson *et al*[48]	21 (15 resected)	DP (9); PP (4); PD (2)	0-NA	NA	NA
Butturini *et al*[49]	5	NA	NA	19 (7-27)	1 (20.0%)
Z'graggen *et al*[3]	2	TP (2)	0-NA	20 (20-40)	2 (100%)
Total	293 (270 resected)	DP (59); TP (32); PD (31); PPPD (16); CP (3); PP (6); enucleation (6)	4 (1.5%)-36 (13.3%)	36.8	72/227 (31.7%)

NA: Not available; PD: Pancreaticoduodenectomy; PPPD: Pylorus-preserving pancreaticoduodenectony; DP: Distal pancreatectomy; CP: Central pancreatectomy; TP: Total pancreatectomy; PP: Partial pancreatectomy.

37 patients were available, 24 with a primary neoplasm of the colon and 11 with a primary rectal cancer. Among these patients, 28 presented with a single pancreatic metastasis and in 9 cases an associated surgical procedure was required for metastatic disease in other sites. There was no perioperative mortality. After pancreatic resection, a recurrence of disease occurred in 19 patients, with a median survival time of 21 mo (range 5-105 mo). Sixteen patients are alive with a median survival time of 12 mo (range 1.5-43 mo), while 5 patients are alive with recurrent disease (6 to 43 mo). It is interesting to note that all patients experienced a relief of symptoms (abdominal pain and obstructive jaundice) after surgical resection of metastases and they remained asymptomatic until recurrence of the disease. It is impossible to establish whether the same results can be achieved in these patients with a more conservative treatment, such as chemotherapy, because of the lack of information regarding the outcome of patients undergoing pancreatic resection and patients undergoing only chemotherapy. Considering the data available in the literature, it seems reasonable to consider surgery for pancreatic metastases from colorectal cancer a palliative treatment. However, it has to be remark that a multidisciplinary approach has to be recommended in the treatment of pancreatic metastates from colorectal cancer, and an aggressive surgical approach may be considered in selected cases, in particular in symptomatic patients with isolated pancreatic metastasis.

Melanoma

Metastases from malignant melanoma can be located in the gastrointestinal tract (50%-60% of cases of malignant melanoma in autopsy series), although the clinical diagnosis occur in only 1.5% to 4.4% of patients[73]. A few cases of long-term survival after radical surgical resection of melanoma metastases in the gastrointestinal tract have been reported[74,75], but the role of surgery in the treatment of pancreatic metastases from melanoma is unknown, due to the lack of data regarding these clinical entities[9,76]. When compared to other primary tumors metastasizing to the pancreas, melanoma seems related to a poor prognosis[28]. In a literature review, we collected a total of 23 reports (19 single-patient reports, 1 with two patients, 3 with more than 2 patients) on surgical treatment of pancreatic metastases from melanoma (Table 3[24,37,58,74,77-95]). Among these patients, 12 had a primary skin melanoma, 6 had an ocular melanoma, 1 had a melanoma of the nasal cavity and in 19 cases the primary

Table 2 Pancreatic resections for metastatic colorectal cancer

Ref.	Year	No	Site of primary	Interval (mo)	Treatment	Survival (mo)	
						Dead	Alive
Roland *et al*[56]	1989	1	Colon	NR	DP		27, AWD
Nakeeb *et al*[57]	1995	1	Colon	34	PD		43, AWD
Harrison *et al*[58]	1997	2	Colon	15	PD	41	
			Colon	15	PD	21	
Inagaki *et al*[59]	1998	1	Rectum	132	DP		8
Yoshimi *et al*[60]	1999	1	Colon	51	PD	24	
Le Borgne *et al*[26]	2000	1	Colon	60	PD	12	
Tutton *et al*[61]	2001	1	Colon	23	DP		12
Torres-Villalobos *et al*[62]	2004	1	Cecum	8	DP		6
Crippa *et al*[24]	2006	1	Colon	7	PPPD	13	
Matsubara *et al*[55]	2007	1	Rectum	28	PD	24	
Eidt *et al*[37]	2007	1	Colon	12	PPPD	105	
Shimoda *et al*[63]	2007	1	Rectum	44	PD	8	
Bachmann *et al*[64]	2007	2	Rectum	24	DP		1.5
			Rectum	30	DP		6
Sperti *et al*[54]	2008	9	Colon (7) Rectum (2)	10-80	PD (2) PPPD (3) DP (4)	525	30, AWD
Reddy *et al*[9]	2008	2	NR	NR	NR		42
Grève *et al*[65]	2008	1	Rectum	54	DP	NR	NR
Gravalos *et al*[66]	2008	1	Cecum	17	DP		12
Machado *et al*[67]	2010	1	Colon	105	DP	9	
Lasithiotakis *et al*[68]	2010	1	Colon	24	PD	27	
Lee *et al*[69]	2010	1	Rectum	24	DP		12
Stoltz *et al*[70]	2011	1	Colon	24	DP		6, AWD
Georgakarakos *et al*[71]	2011	1	Colon	12	PD		6
Tanemura *et al*[72]	2012	2	Rectum	72	MSPP		16
			Rectum	84	DP		6
Niess *et al*[29]	2013	2	Colon	0	PPPD	68	21, AWD
			Colon	14	DP		
Total		37	Colon (24) Rectum (11) NR (2)	24 (median)	PD (11) DP (17) PPPD (6) MSPP (1)	21 (median)	12 (median)

NR: Not reported; DP: Distal pancreatectomy; PD: Pancreaticoduodenectomy; PPPD: Pylorus-preserving pancreaticoduodenectomy; MSPP: Middle-segment-preserving pancreatectomy; SMV: Superior mesenteric vein; AWD: Alive with disease.

site of melanoma was unknown. No perioperative mortality was reported. Twenty patients died of recurrent disease: the median survival time of these patients was 10 mo (range 3-25 mo). Thirteen patients are alive at 6 to 108 mo (median 16 mo); 2 patients were alive, with recurrence, at 8 and 12 mo respectively. Although malignant melanoma is associated with a poor prognosis and the role of surgery seems limited to palliation, some cases of a prolonged survival after surgical removal of melanoma metastases have been reported[74] and, when possible, surgical resection seems to be the most effective therapeutic option available today[96,97]. However, there are no sufficient data in the literature to compare patients treated with only conservative management (chemotherapy) with surgical resected patients. Therefore surgical resection for pancreatic metastases from melanoma should be considered a palliative treatment, to be taken in account in pancreatic isolated lesions as a part of the multimodality treatment of this clinical entity.

Breast carcinoma

Pancreatic metastases from breast cancer are rare, with a reported rate of 13% in an autopsy series[98]. Metastatic breast cancer is usually a widespread disease, with isolated pancreatic lesions being an occasional event. In a literature review, we selected 16 studies regarding patients undergoing surgery for pancreatic metastases from breast cancer (Table 4[9,24,26,40,57,99-110]). Breast cancer that metastasize to the pancreas may have a long latency period between the primary tumor diagnosis and the metastasis occurrence (median 39.5 mo, range 0-216). Solitary pancreatic metastasis was present in 17 patients, and 1 underwent also a subtotal gastrectomy for extrapancreatic involvement. There was no perioperative mortality. Five patients died of recurrent disease: the survival time was available in only three of these patients and the median was 26 mo (range 7-36 mo). Fourteen patients are alive at 5 to 80 mo (median 19), although 5 patients had a short follow-up (up to 12 mo) and in one patients follow-up time is not reported; 3 patients were alive, with recurrence, at 11 to 48 mo. All patients experienced a relief of symptoms (abdominal pain and obstructive jaundice) after surgical resection of metastases and they remained asymptomatic until recurrence of the disease. Masetti *et al*[28]

Table 3 Pancreatic resections for metastatic melanoma

Ref.	Year	No	Interval (Yr)	Primary site	Surgery	Follow-up (mo)	Outcome
Dasgupta *et al*[77]	1964	1	2	Skin	DP + duodenal resection	10	DOD
Johansson *et al*[78]	1970	1	12	Ocular	PD	11	ANED
Lasser *et al*[79]	1990	1	8	Skin	PD	10	ANED
Bianca *et al*[80]	1991	1	NA	NA	PD	12	AWD
Brodish *et al*[81]	1993	1	34	Skin	DP	8	AWD
Harrison *et al*[58]	1997	1	NR	NA	PD	108	ANED
Medina-Franco *et al*[82]	1999	1	NA	NA	PPPD	6	DOD
Wood *et al*[74]	2001	8	NA	NA	PD	37.5%[1]	DOD
Hiotis *et al*[83]	2002	1	NR	NR	PD	NR	DOD
Camp *et al*[84]	2002	1	6	Ocular	DP	20	ANED
Nikfarjam *et al*[85]	2003	2	12, 13	Ocular	PPPD, TP	6, 7	ANED
Carboni *et al*[86]	2004	1	9	Skin	PD	4	DOD
Crippa *et al*[24]	2006	1	2.8	Skin	PPPD	14	DOD
Belágyi *et al*[87]	2006	1	6	Skin	Enucleation	4	DOD
Edit *et al*[37]	2007	4	3, 4, 4, 14	NA	PPPD (4)	12, 25 30, 76	DOD ANED
Vagefi *et al*[88]	2009	1	28	Ocular	DP	NR	NR
Sperti *et al*[89]	2009	1	3	NA	DP	24	DOD
He *et al*[90]	2010	1	5	Ocular	DP	25	ANED
Lanitis *et al*[91]	2010	1	5	Skin	PD	96	ANED
Moszkowicz *et al*[92]	2011	1	15	Skin	PD	NA	NA
Portale *et al*[93]	2011	1	7	Skin	DP	NA	ANED
Goyal *et al*[94]	2012	5	3, 22, ?, 5, ?	Skin (3), NA (2)	PPPD (4),DP(1)	15, 3, 11.4, 4.5, 25	DOD
Sugimoto *et al*[95]	2013	1	1	Nasal	DP	10	DOD
Total		38	6 (median)	Skin = 12; Ocular = 6; Nasal = 1; NA = 18; NR = 1	PD (16), DP (9), PPPD (11), TP (1), Enucleation (1)	11, 7 (median)	

[1]5 years survival rate. NR: Not reported; NA: Not available; DP: Distal pancreatectomy; PD: Pancreaticoduodenectomy; PPPD: Pylorus-preserving pancreaticoduodenectomy; MSPP: Middle-segment-preserving pancreatectomy; SMV: Superior mesenteric vein; DOD: Dead of disease; ANED: Alive not evidence of disease; AWD: Alive with disease.

analysing the prognostic factors in metastatic tumors to the pancreas, found at univariate survival analysis a 2-years probability of survival of 57.1% in pancreas metastases from breast cancer and a 5-years probability of survival of 34.3%. Even in the case of pancreatic metastases from breast cancer it is impossible to establish the course of the disease without surgical resection and to assess the real benefit in survival after metastasectomy. However, in selected patients with limited pancreatic disease, surgical resection could have a palliative role in association with chemotherapy, hormonal therapy and radiation therapy in the multimodality treatment of metastatic breast carcinoma.

Lung cancer

Lung cancer metastasize to many site, but most frequently to bone, liver and adrenal glands[111,112]. Isolated pancreatic metastases from lung cancer are extremely rare[76] and they are usually metachronous lesions, identified at follow-up investigation. The few reports available in the literature show that small cell lung cancer (SCLC) represents the most typical histological subtype metastasizing to the pancreas[113].

The usefulness of surgical resection for pancreatic metastasis from lung cancer is difficult to assess because of the rarity of this type of lesion. Additionally, most cases of pancreatic metastasis from lung cancer are unresectable at the time of diagnosis because the disease is already widespread. Z'graggen *et al*[3] and Moussa *et al*[40] reported four patients each with secondary metastasis from lung cancer (including small cell lung cancer): there were no resectable cases mainly due to local invasion and metastases to other organs. Hiotis *et al*[83] reported three cases of pancreatic resections for metastatic lung cancer, with a poor long-term survival after surgery. In a recent review of the literature, Reddy *et al*[9] reported pancreatic resections from lung cancer as having the worst outcome when compared to other primary tumors type metastatic to the pancreas. In a literature review, we selected 12 studies reporting surgical resection for pancreatic involvement from lung cancer (Table 5[24,26,57,68,82,83,102,114-118]). Among these patients, in 10 cases the primary lung cancer was a NSCLC, 1 case was a SCLC and in the last patient the primary lung cancer is not specified. One patient died after surgical resection. Five patients died of recurrent disease, with a median survival time of 7 mo (range 3-14 mo). Six patients are alive with a median survival time of 19 mo (range 6-24 mo). In all cases, preoperative symptoms (obstructive jaundice and abdominal pain) disappeared after surgery. Pancreatic metastases from lung cancer have a poor prognosis and treatment options for metastatic lung cancer lesions to the pancreas are mainly

Table 4 Pancreatic resections for metastatic breast cancer

Ref.	Yr	No	Interval (mo)	Treatment	Survival (mo)	
					Dead	Alive
Bednar *et al*[99]	2013	1	216	PD		48 mo, AWD
Razzetta *et al*[100]	2011	1	0	PD		11 mo, AWD
Bonapasta *et al*[101]	2010	1	23	PD	36	
Mourra *et al*[102]	2010	1	9	DP		20 mo
Sweeney *et al*[103]	2009	1	60	DP		NA
Reddy *et al*[9]	2008	1	NR	NR	NR	13 mo
Jiménez-Heffernan *et al*[104]	2006	1	0	PD		10 mo
Tohnosu *et al*[105]	2006	1	52	DP		5 mo
Crippa *et al*[24]	2004	3	60/36/84	PPPD (3)	26	21AWD/37
Moussa *et al*[40]	2004	1	45	TP	7	
Minni *et al*[106]	2004	1	26	enucleation		80
Ogino *et al*[107]	2003	1	72	PD	Dead (-)	
Le Borgne *et al*[26]	2000	1	0	PD		12
Nomizu *et al*[108]	1999	1	80	PD		18
Mehta *et al*[109]	1997	1	36	PD		27
Nakeeb *et al*[57]	1995	1	19	PD		12
Azzarelli *et al*[110]	1982	1	43	PD		72
Total		19	39.5 mo (median)	PD (10) DP (3) PPPD (3) Enucleation (1) TP (1)	26 (median)	19 (median)

NR: Not reported; DP: Distal pancreatectomy; NA: Not available; PD: Pancreaticoduodenectomy; PPPD: Pylorus-preserving pancreaticoduodenectomy; TP: Total pancreatectomy; AWD: Alive with disease.

palliative.

Sarcoma

Metastatic sarcoma has generally a poor survival, and radical surgical represent the only therapeutical chance for these patients. Isolated pancreatic involvement by sarcomas is rarely encountered: in a recent experience Yoon *et al*[119] reported only 2 cases (4%) of sarcomas among 53 patients with pancreatic metastases collected at their Institution. So, the outcomes for patients with metastatic sarcoma who did or did not pancreatic resection are unknown[53]. In their review, Reddy *et al*[53] collected only 10 patients with isolated pancreatic metastasis with a median survival of 40 mo and 5-year survival of 14%. Even if pancreatic metastases from sarcoma seem related with a modest survival, the few data available does not allow to draw any definitive conclusion. Recently, Robert *et al*[120] reported a case of leiomyosarcoma metastatic to the pancreas and collected 17 of the such cases published in the Literature. Clinical details were available in only 8 reports, and 7 patients underwent pancreatic resection: 5 patients were alive (one with disease) and 2 died, with a median survival time of 23 mo. As for other cancers, resection of pancreatic metastases from sarcoma is substantially justified in individual basis.

In recent years, an increased number of surgical resections for pancreatic metastases has been performed in high-volume centers. It seems reasonable that resection is indicated for an isolated and resectable metastasis in a patient fit to tolerate pancreatectomy, evaluating each single case on an individual basis and with a multidisciplinary approach.

The type of surgical procedure is another controversial aspect in pancreatic metastases. Standardized pancreatic resection adapted to the location of the tumor, in terms of partial pancreaticoduodencectomy, distal pancreatectomy, and total pancreatectomy, is generally recommended for the management of isolated pancreatic metastases. Bassi *et al*[17] observed a high rate of pancreatic recurrences after atypical resections and recommended standard radical resection. Considering the high frequency of multiple metastases, a recurrence after surgical resection could be related to multifocality of the tumor rather than to an atypical surgical procedure[18]. Since pancreatic metastases is often multifocal, partial pancreatectomies require thorough exploration of the pancreatic remnant by palpation and ultrasound. Intraoperative ultrasound is a very useful device: it guides the surgeon in choosing the most appropriate surgical procedure by defining the presence of multiple pancreatic lesions and the proximity of the metastasis to the Wirsung duct[18]. Surgical strategy should be tailored on each single case, in order to achieve an R0 resection and ensuring the absence of further disease in the pancreatic parenchyma. Surgical resection may be considered also in selected cases of extrapancreatic disease, if technically feasible[16]. The effectiveness of resection for pancreatic metastases is mainly dependent on the tumor biology of the primary cancer.

The benefit of metastasectomy in terms of patient survival has been observed for metastases from RCC, while for other primary tumors the role of surgery is mainly palliative. Patients with pancreatic metastases

Table 5 Pancreatic resections for metastatic lung cancer

Ref.	No	Interval (mo)	Type of primary	Treatment	Survival (mo)	
					Dead	Alive
Igai *et al*[114]	1	60	NSCLC	PD		6
Lasithiotakis *et al*[68]	1	6	NSCLC	PD	/	/
Mourra *et al*[102]	2	0, 10	NSCLC	DP (2)	10	20
Wilson *et al*[115]	1	NA	NSCLC	PD		22
Mori *et al*[116]	1	22	NSCLC	PPPD		24
Pericleous *et al*[117]	1	0	NSCLC	PPPD		18
Crippa *et al*[24]	1	5	NSCLC	PPPD	14	
García Vidal *et al*[118]	1	0	NSCLC	PD		NA
Hiotis *et al*[83]	1	NA	NA	DP	DOD	
Le Borgne *et al*[26]	1	0	SCLC	PD	4	
Medina-Franco *et al*[82]	1	17	NSCLC	PD		12
Nakeeb *et al*[57]	1	8	NSCLC	PD	3	
Total	13	6 (median)	NSCLC = 10 SCLC = 1	PD (7) DP (3) PPPD (3)	7 (median)	19 (median)

NSCLC: Non-small cell lung cancer; SCLC: Small cell lung cancer; NA: Not available; DP: Distal pancreatectomy; PD: Pancreaticoduodenectomy; PPPD: Pylorus-preserving pancreaticoduodenectomy; DOD: Dead of disease.

from RCC represent a favourable subgroup and surgical resection is recommended for these patients, whenever possible. However, a multidisciplinary approach has to be recommended and further studies are needed to establish the way to combine surgery with medical treatment in the different periods of the disease.

Considering the data available in the literature, it seems reasonable to consider surgery for pancreatic metastases from colorectal cancer a palliative treatment. However, an aggressive surgical approach may be considered in selected cases, in particular in symptomatic patients with isolated pancreatic metastasis.

Resection of melanoma metastatic to the pancreas appears to be only a palliative procedure. However, surgical resection may be considered in limited pancreatic disease with palliative intent. Even in the case of pancreatic metastases from breast cancer it is impossible to establish the course of the disease without surgical resection and to assess the real benefit in survival of the metastasectomy. However, in selected patients with a limited pancreatic disease, surgery may play a role in conjunction with chemotherapy, hormonal therapy and radiation therapy in the multimodality treatment of metastatic breast carcinoma. Solitary pancreatic metastases from lung cancer have a poor prognosis and treatment options for metastatic lung cancer lesions to the pancreas are mainly palliative. Finally, resection of pancreatic metastases from sarcoma is substantially justified in individual basis.

CONCLUSION

Pancreatic metastases, although uncommon, are an increasing clinical entity. Surgical resection is often advocated when the lesion is single and for patients fit to perform a pancreatectomy. The usefulness of pancreatic resection is mainly linked to the biology of the primary tumor metastasizing to the pancreas. The benefit of metastasectomy in terms of patient survival has been observed for metastases from RCC, while for other tumors the role of surgery is mainly palliative. In fact, from our data and from a review of the literature, pancreatic surgery for metastases from colorectal cancer and melanoma may be considered for palliation, even if in selected cases surgical resection can be advocated in the multimodality treatment of metastatic colorectal cancer. Even in the case of pancreatic metastases from breast cancer, an aggressive surgical approach appears useful for good palliation in selected patients with a limited pancreatic disease. Patients with solitary metastases from lung cancer have a poor outcome and do not benefit from surgical resection. Finally, resection of pancreatic metastases from sarcoma is substantially justified only in very selected patients.

Patients with pancreatic metastases should be evaluated with a multidisciplinary approach, being surgery part of the multimodality treatment of these clinical entities. Further studies are needed to establish the way to combine surgery with medical treatments in the different metastatic diseases to the pancreas.

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MINIREVIEWS

Multimodality management of resectable gastric cancer: A review

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Abstract

Adenocarcinoma of the stomach carries a poor prognosis and is the second most common cause of cancer death worldwide. It is recommended that surgical resection with a D1 or a modified D2 gastrectomy (with at least 15 lymph nodes removed for examination) be performed in the United States, though D2 lymphadenectomies should be performed at experienced centers. A D2 lymphadenectomy is the recommended procedure in Asia. Although surgical resection is considered the definitive treatment, rates of recurrences are high, necessitating the need for neoadjuvant or adjuvant therapy. This review article aims to outline and summarize some of the pivotal trials that have defined optimal treatment options for non-metastatic non-cardia gastric cancer. Some of the most notable trials include the INT-0116 trial, which established a benefit in concurrent chemoradiation and adjuvant chemotherapy. This was again confirmed in the ARTIST trial, especially in patients with nodal involvement. Later, the Medical Research Council Adjuvant Gastric Infusional Chemotherapy trial provided evidence for the use of perioperative chemotherapy. Targeted agents such as ramucirumab and trastuzumab are also being investigated for use in locally advanced gastric cancers after demonstrating a benefit in the metastatic setting. Given the poor response rate of this difficult disease to various treatment modalities, numerous studies are currently ongoing in an attempt to define a more effective therapy, some of which are briefly introduced in this review as well.

Key words: Neoadjuvant chemotherapy; Adjuvant chemotherapy; Adjuvant chemoradiation; Gastric cancer; Gastric adenocarcinoma

Core tip: Gastric adenocarcinoma is a difficult disease to treat. Surgical resection is the definitive therapy but recurrences are frequent. The use of a multidisciplinary approach to treatment decision-making is imperative. Surgical resection should be an R0 resection (with clear macroscopic and microscopic margins) and at least a D1 lymphadenectomy with a minimum of 15 lymph nodes sampled in the United States and a D2 lymphadenectomy elsewhere. Perioperative chemotherapy is a reasonable option based on the Medical Research Council Adjuvant Gastric Infusional Chemotherapy trial. In patients who are evaluated after resection, adjuvant chemoradiation adds important survival benefit. Other options include adjuvant S-1 in Asian patients, capecitabine/oxaliplatin, and capecitabine/cisplatin.

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INTRODUCTION

Adenocarcinoma of the stomach is one of the most common malignancies in the world, ranking fifth after

lung, breast, colorectal, and prostate. According to the World Health Organization, 952000 new cases were diagnosed in 2012 alone, with more than 70% of all cases occurring in developing countries[1]. In the United States, an analysis using the Surveillance Epidemiology and End Results database of the National Cancer Institute found an increase in overall incidence of adenocarcinoma of the esophagus and the gastric cardia from 13.4 per million in 1973 to 51.4 per million in 2009[2]. It is also the second most common cause of cancer death as of 2010. There is a significant disparity in the incidence and survival rates between the Asian and Western countries. For example, the overall 5-year survival worldwide was about 20% according to a report in 2008 but more than 70% in Japan for resectable disease. Such a dramatic difference maybe due to the implementation of screening programs in Japan where there is a higher incidence of gastriccancer resulting in detection of disease at earlier stages. In contrast, patients in the United States are usually diagnosed later in stage as routine screening for gastric cancer is not recommended owing to cost ineffectiveness[3]. The survival benefit may also be related to a more frequent use of second-line chemotherapy in Asian countries, most commonly irinotecans and taxanes, compared to the West[4, 5].

While gastric adenocarcinoma obviously includes tumors arising from the stomach, the classification of tumors of the gastroesophageal junction (GEJ) has been a topic of debate. The most widely used classification was proposed by Rüdiger Siewert *et al*[6] in 2000: type I tumors are tumors in the distal esophagus and may extend to the GEJ from above, type Ⅱ tumors are adenocarcinomas of the cardia, arising at the GEJ, and type Ⅲ tumors are cancers that originated from below the cardia and extend to the GEJ and distal esophagus from below. It is also noted that the biologies of these distinct types of GEJ tumors are very different. Type I cancers are mostly associated with intestinal metaplasia and history of gastroesophageal reflux disease. On the other hand, types Ⅱ and Ⅲ cancers resemble proximal gastric cancer and have lymphatic spread preferentially to the celiac axis[6,7]. The American Joint Committee on Cancer (AJCC) updated the staging of stomach adenocarcinoma in the 7th edition to include cancers of the GEJ arising more than 5 cm distally of the GEJ or within 5 cm of the GEJ but without extension to the esophagus or GEJ[8]. This distinction is important because many of the clinical trials included cancers of the GEJ in addition to cancers of the stomach. More importantly, cancers of the GEJ as described above behave similarly compared to gastric cancer and are treated as such.

Currently, surgical resection is the only curative mode of treatment for non-metastatic gastric adenocarcinoma. However, median survival with surgery alone, historically, was poor. Patients who had undergone resection are prone to suffer from locoregional or distant recurrences of their disease. As a result, neoadjuvant and adjuvant therapies aimed at the eradication of micrometastases were studied in an attempt to reduce recurrence and prolong survival. This review article aims to outline some of the pivotal data that led to current clinical practices in resectable gastric cancer. It also briefly introduces ongoing trials in a global effort to improve overall survival for this difficult disease. Data presented in this review article were retrieved using a PubMed search with the key words "adjuvant," "neoadjuvant," "perioperative therapy," and "resectable gastric cancer."

CURATIVE RESECTION

Though this review aims to summarize available data in medical treatment of resectable gastric cancer, it is important to discuss surgical management given its central role in overall management. Controversies surround the surgical management of gastric cancer. In 1999, Bozzetti *et al*[9] found no difference in survival between total and subtotal gastrectomies but that subtotal gastrectomy was associated with improved nutritional status and quality of life. With the advancement of laparoscopic techniques, laparoscopic gastrectomy was found to have similar outcomes but with fewer complications compared to open gastrectomy in meta-analyses and case-control studies[10-13]. Furthermore, a resection margin of 1 mm was found to be sufficient as long as the resection margins were free of tumor[12].

The depth of lymphadenectomy has been a topic of debate as well. A D1 dissection involves a gastrectomy and the removal of the greater and lesser omental lymph nodes. A D2 dissection involves the above plus the removal of all lymph nodes along the left gastric artery, common hepatic artery, celiac artery, splenic hilum and splenic artery. The D1 dissection was traditionally favored in the West, specifically in the United States, whereas D2 resection was preferred in the East[14] and Europe. This discrepancy was based on early randomized trials that failed to show a survival benefit with D2 lymphadenectomy[15,16]. Subsequent studies showed that D2 resection indeed offered a survival benefit, prompting a change in practice. Recently, Shrikhande *et al*[17] established the non-inferiority of perioperative gastrectomy with D2 lymphadenectomy for locally advanced resectable gastric adenocarcinoma when combined with neaoadjuvant chemotherapy. More importantly, half of those patients who achieved a pathologic response were found to have lymph node involvements, arguing for the necessity of D2 gastrectomy[17]. A randomized trial comparing D1 and D2 dissections found that there was no difference in overall 5-year survival between the two practices. However, subgroup analyses suggest that D1 resection may be beneficial for those with pT1 disease while a trend towards improved survival was seen with D2 lymphadenectomy in patients with nodal involvement[18]. Based on some of these trials in addition to other clinical data, the National Comprehensive Cancer Network guidelines currently recommends a D1 or a modified D2 gastrectomy with at least 15 lymph nodes removed for examination in

the United States, though noting that D2 lymphadenectomies should be performed at experienced centers[19].

NEOADJUVANT CHEMOTHERAPY

Neoadjuvant treatment has the appeal of allowing for a more complete surgical resection while assessing for response to chemotherapy and risk for recurrence. However, robust data to support use of neoadjuvant therapy are limited at this time. Schuhmacher *et al*[20] reported data from the European Organisation for Research and Treatment of Cancer 40954 trial comparing neoadjuvant cisplatin, folinic acid, and infusional fluorouracil with surgery alone. A total of 144 patients with locally advanced adenocarcinoma of the stomach and GEJ were recruited and randomized. Those assigned to chemotherapy received 48-d cycles of neoadjuvant biweekly cisplatin, weekly L-folinic acid and fluorouracil for 2 cycles. The study was closed prematurely due to poor accrural. Only 62.5% of patients assigned to the chemotherapy arm completed 2 cycles of treatment.

Median follow-up was about 4 years. Preoperative chemotherapy reduced tumor size and nodal involvement compared to surgery alone. Given the low accrural, this study was ultimately underpowered at 25%. Progression-free survival had a hazard ratio of 0.76 but was not statistically significant (95%CI: 0.49 to 1.16, $P = 0.2$). The 2-year survival rates were 72.7% in the chemotherapy arm and 69.9% in the surgery only arm. The hazard ratio for overall survival was 0.84 in favor of chemotherapy, though it was not a statistically significant finding (95%CI: 0.52 to 1.35, $P = 0.466$). The authors noted that while this was a negative study with a small sample size, the rate of R0 resection was higher in the group that received neoadjuvant chemotherapy at 81.9%, compared to 66.7% in the group that did not ($P = 0.036$)[20]. Whether this difference would have translated into a benefit in progression-free survival or overall survival remains unanswered.

Additional albeit limited trial data emerged recently in attempts to further characterize the use and benefits of neoadjuvant chemotherapy. A small randomized, double-blinded controlled trial from Tehran found similar survival rates after a follow-up period of about 10 mo when comparing use of preoperative docetaxel, cisplatin, and 5-fluorouracil (DCF) followed by surgery with surgery alone[21]. In a recent phase Ⅱ study, the use of neoadjuvant paclitaxel and cisplatin was found to provide a pathologic response of 34.6% and a 3-year overall survival of 41.5% (95%CI: 27.4% to 55.0%)[22]. A small non-randomized study from China compared the use of epirubicin, oxaliplatin, and capecitabine (EOX) with 5-fluorouracil, leucovorin, and oxaliplatin (FOLFOX). An improved pathologic response was found with use of EOX. This study, however, enrolled 87 patients in the FOLFOX arm and only 26 patients in the EOX arm[23].

Given the paucity and variability of information, systemic reviews were conducted to attempt to clarify the role of neoadjuvant chemotherapy. A meta-analysis was performed investigating the effectiveness of 5-fluorouracil-based chemotherapy in the neoadjuvant setting. Seven randomized controlled trials were included for analysis with a total of 1249 patients. The results showed that neoadjuvant chemotherapy improved overall survival with an odds ratio of 1.40 (95%CI: 1.11 to 1.76, $P = 0.0005$). The 3-year progression-free survival was also higher in the chemotherapy group at 37.7% compared to 27.3% in the control group, odds ratio of which was 1.62 (95%CI: 1.21 to 2.15, $P = 0.001$). There was no difference in perioperative mortality or complication rates between the two groups. Combination chemotherapy was superior to monotherapy. Additionally, intravenous administration of chemotherapy was found to have a greater impact than oral administration. Finally, it demonstrated a preference in Western countries for neoadjuvant treatment compared to Asian countries[24].

On the other hand, Liao *et al*[25] did not find an improvement in overall survival or R0 resection with use of neoadjuvant therapy. A meta-analysis of 6 randomized, controlled trials with 781 patients was conducted. The odds ratio was 1.16 for overall survival with use of neoadjuvant chemotherapy (95%CI: 0.85 to 1.58, $P = 0.36$) and 1.24 for R0 resection (95%CI: 0.78 to 1.96, $P = 0.36$)[25], neither of which were statistically significant. Currently, available data further illustrates the controversy in defining the optimal neoadjuvant treatment.

PERIOPERATIVE CHEMOTHERAPY

The Medical Research Council Adjuvant Gastric Infusional Chemotherapy (MAGIC) Trial in 2006 established the role of perioperative chemotherapy for resectable gastroesophageal cancer as the standard of care. A total of 503 treatment-naïve patients with adenocarcinoma of the stomach or lower third of the esophagus were randomized to receive perioperative epirubicin, cisplatin, and infused fluorouracil (ECF) or surgery alone. The trial was initially designed to recruit gastric adenocarcinomas but was extended to include tumors of the GEJ due to its increased incidence. Patients had stage Ⅱ and Ⅲ disease or locally advanced but inoperable disease.

Two hundred and fifty patients were randomized to receive 3 cycles of preoperative epirubicin (50 mg/m^2 on day 1), cisplatin (60 mg/m^2 on day 1), and fluorouracil (200 mg/m^2 daily) for 21 d, followed by surgical resection and 3 additional cycles of ECF. A total of 215 patients, 86% of those randomized to the perioperative chemotherapy arm, completed chemotherapy; 41.6% of these patients completed all 6 cycles of chemotherapy. Median follow-up was about 4 years. Preoperative chemotherapy significantly reduced tumor size at time of resection with a median maximum diameter of 3 cm (compared to 5 cm in those without chemotherapy, $P < 0.001$). There was also more T1 and T2 tumors as well as N0 and N1 disease in the group exposed to chemotherapy. Five-year survival rates were 36.3% in the perioperative chemotherapy arm and 23% in the surgery arm with an overall sur-

vival hazard ratio of 0.75 (95%CI: 0.60 to 0.93, $P = 0.009$). Progression-free survival was also improved with chemotherapy with a hazard ratio of 0.66 (95%CI: 0.53 to 0.81, $P < 0.0019$). Local recurrence was noted in 14.4% of patients in the perioperative chemotherapy group and in 20.6% in the surgery group. Distant metastases were also less frequent in those who received chemotherapy (24.4% *vs* 36.8%)[26]. The benefits of this regimen was confirmed in 2013 when Mirza *et al*[27] found an improvement in survival when patients completed both the pre- and postoperative cycles.

In 2007, the results for the FNLCC ACCORD07-FFCD 9703 trial were presented at the annual American Society of Clinical Oncology meeting and later published in 2011. A total of 224 patients with adenocarcinoma of the stomach or GEJ were randomized to receive 2-3 cycles of fluorouracil at 800 mg/m^2 for days 1-5 and cisplatin 100 mg/m^2 on day 1, for a 28-d cycle followed by surgery and postoperative chemotherapy for an additional 3-4 cycles or surgery alone. The planned maximum cycles were set at 6. The trial was closed early as a result of accrural difficulties.

The median follow-up was 5.7 years. In the chemotherapy arm, 97% of patients received at least 1 cycle of preoperative chemotherapy, 87% received at least 2 cycles. Of these, 50% went on to receive post-operative chemotherapy. R0 resection rate was 84% in the chemotherapy group compared to 74% in the surgery group ($P = 0.04$). There was a trend towards less nodal involvement at time of surgery in the chemotherapy group (67% *vs* 80%, $P = 0.054$) but the sizes of tumors at resection were similar in both groups. Five-year survival was 38% (95%CI: 29% to 47%) in the chemotherapy group and 24% (95%CI: 17% to 33%) in the surgery group. Five-year disease-free survival was also significantly improved with chemotherapy at a rate of 34% (95%CI: 26% to 44%) compared to 19% (95%CI: 13% to 28%). Furthermore, the chemotherapy arm also offered improved overall survival with a hazard ratio of 0.69 (95%CI: 0.50 to 0.95, $P = 0.02$) and disease-free survival with a hazard ratio of 0.65 (95%CI: 0.48 to 0.89, $P = 0.003$).

It is important to note, however, that this study was originally designed to include patients with cancer of the esophagus and was only extended to include cancer of the stomach in 1998. Consequently, 64% of accrued patients had disease of the GEJ while only 25% had gastric carcinoma. In a multivariate analysis, it was noted that preoperative chemotherapy and tumor site at the GEJ were significant prognostic factors for overall survival, $P = 0.01$ and $P < 0.01$, respectively. The other pathologies were not noted to have a statistically significant benefit when analyzed separately because of small sample sizes[28,29].

In a small non-randomized study, the use of perioperative FOLFOX was compared with adjuvant FOLFOX. A total of 73 patients with resectable T3 and T4 gastric adenocarcinoma were recruited between December 2001 and September 2005, 33 of which were assigned to the perioperative arm while 37 patients were assigned to the adjuvant arm. Those receiving perioperative chemotherapy received 3-wk cycles of FOLFOX for 2-4 cycles, followed by surgery and further chemotherapy for a total of 6 cycles. Those allocated to the adjuvant arm received the same FOLFOX regimen for a total of 6 cycles. The median follow-up duration was 53 mo. The 4-year overall survival was 78% (95%CI: 64% to 92%) in the perioperative chemotherapy group compared to 51% (95%CI: 35% to 67%, $P = 0.031$) in the adjuvant group. The 4-year disease-free survival was 78% (95%CI: 64% to 92%) and 48% (95%CI: 32% to 64%, $P = 0.022$), respectively[30]. While this was a very small, non-randomized study, it provided evidence for further investigational efforts to evaluate the role of FOLFOX in a perioperative setting.

Finally, the use of perioperative chemotherapy, with or without radiation, was confirmed as advantageous compared to surgery alone in a Cochrane database meta-analysis of randomized controlled trials. The hazard ratio with use of chemotherapy was 0.81 (95%CI: 0.73 to 0.89), which corresponded to a 5-year relative survival increase of 19% and an absolute increase of 9%[31].

ADJUVANT CHEMORADIATION

In 2001, Macdonald *et al*[32] published clinical results from the INT-0116 (Intergroup 0116) study evaluating effects of adjuvant chemoradiation using concurrent fluorouracil and leucovorin followed by 2 cycles of fluorouracil and leucovorin after completion of radiation as compared to surgery alone. The regimen used is now commonly known as the Macdonald regimen. This study also changed the standard of care for gastric adenocarcinoma. It recruited 603 patients between 1991 and 1998 with stages IB to IV(M0) gastric or gastroesophageal adenocarcinoma. Gastric primaries comprised of about 80% of total recruited patients. Sixty-four percent of those randomized to chemoradiation completed treatment. Median follow-up was 5 years with median survival of 36 mo in the chemoradiation group and 27 mo in the control group. Three-year survival rates were 50% in the chemoradiation arm and 41% in the surgery arm, with a hazard ratio of 1.35 (95%CI: 1.09 to 1.66, $P = 0.005$) in the surgery arm. The median progression-free survival was 30 mo with adjuvant treatment compared to 19 mo without, which translated to three-year rate of progression-free survival of 48% and 31%, respectively. One of the criticisms of this trial was that more than half of the patients had less than D1 resections. It was possible that the adjuvant treatment acted to compensate for the suboptimal surgery. The effect of adjuvant radiotherapy in setting of D2 resections remains unclear from this data set[32].

After median follow-up of 10.3 years, an update to the INT-0116 trial was presented in 2012. The hazard ratio for progression-free survival was 1.51 (95%CI: 1.25 to 1.83, $P < 0.001$) and 1.32 (95%CI: 1.10 to 1.60, $P = 0.0046$) for overall survival without the addition of

chemoradiation. Median progression-free survival was 27 mo for adjuvant therapy compared to 19 mo without ($P < 0.001$). Median overall survival was 35 mo with additional treatment compared to 27 mo without ($P = 0.0046$). There was no notable long term adverse effect found. This update confirmed earlier findings that additional adjuvant chemoradiation offered significant benefit in gastric cancer[33].

With the approval of capecitabine in 1998 for breast cancer and subsequently colorectal cancer, a new oral option became available. Using this new oral fluorouracil prodrug, the ARTIST (Adjuvant Chemoradiation Therapy in Stomach Cancer) trial expanded on the idea of adjuvant chemoradiation. It compared adjuvant capecitabine and cisplatin with capecitabine, cisplatin and concurrent capecitabine chemoradiation. From 2004 to 2008, 458 patients with adenocarcinoma of the stomach who had undergone an R0 gastrectomy with at least D2 lymph node dissection were randomized. Those assigned to the chemotherapy arm received 6 cycles of capecitabine (1000 mg/m^2 twice daily on days 1-14) and cisplatin (60 mg/m^2 on day 1) every 3 wk. Those assigned to the chemoradiation received 2 cycles of the same doses of capecitabine and cisplatin, followed by concurrent capecitabine (825 mg/m^2 twice daily) and radiation, followed by 2 additional cycles of capecitabine and cisplatin in 3-wk cycles.

Median duration of follow-up was 53.2 mo. Treatments were completed by 75.4% of those randomized to the chemotherapy arm and 81.7% of those assigned to the chemoradiation arm. Three-year disease-free survival rates were 78.2% in the concurrent chemoradiation group and 74.2% in the chemotherapy alone group ($P = 0.0862$). While this was not statistically significant, a subgroup analysis found a statistically significant improvement in 3-year disease-free survival in patients with nodal involvement using chemoradiation (77.5% *vs* 72.3%, $P = 0.0365$), which corresponded to a hazard ratio of 0.6865 (95%CI: 0.4735 to 0.9952, $P = 0.0471$). Overall survival data had not matured at time of publication. It should be noted that while disease-free survival was improved with the addition of radiation, the rate of locoregional recurrence and distant metastases were not different between the two study groups[34].

CALGB 80101, a US Intergroup study, compared the INT-0116 protocol regimen (bolus FU and leucovorin with FU plus concurrent RT) versus postoperative ECF before and after FU plus concurrent RT in 546 patients with completely resected gastric or GEJ tumors that extended beyond the muscularis propria or were node positive[35]. The fraction of enrolled patients with GEJ versus gastric primary tumors was not reported. In a preliminary report presented at the 2011 meeting of the American Society of Clinical Oncology, patients receiving ECF had lower rates of diarrhea, mucositis, and grade 4 or worse neutropenia. Overall survival, the primary endpoint, was not significantly better with ECF (at three years, 52% *vs* 50% for ECF and FU/LV, respectively). The trial was not adequately powered to assess non-inferiority. The location of the primary tumor GEJ *vs* proximal versus distal stomach did not have any effect on treatment outcome.

A meta-analysis also confirmed the utility of adjuvant chemoradiation in resectable gastric adenocarcinoma after an R0 resection[36].

ADJUVANT CHEMOTHERAPY

As perioperative and adjuvant chemoradiation became widely accepted, the benefit of adjuvant chemotherapy was also investigated. The Adjuvant Chemotherapy Trial of S-1 for Gastric Cancer (ACTS-GC) trial sought to answer this question. S-1 is an oral dihydropyrimidine dehydrogenase inhibitory fluoropyrimidine combination of tegafur, gimeracil, and oteracil. Once ingested, tegafur is converted *in vivo* to fluorouracil. This was a phase Ⅲ, randomized study that recruited 1059 patients with stage Ⅱ or Ⅲ adenocarcinoma of the stomach from 2001 to 2004. All patients underwent a D2 gastrectomy with an R0 resection. Those patients assigned to adjuvant therapy received S-1 in 80, 100, or 120 mg daily doses, estimated based on body surface area, for 4 wk with 2 wk of rest for 1 year.

The study initially found, after a median follow up of 3 years, that the 3-year overall survival was 80.1% in the S-1 group compared to 70.1% in the surgery alone group. The hazard ratio was 0.68 (95%CI: 0.52 to 0.87, $P = 0.003$). The investigators performed an updated analysis of the results after 5 years of follow-up in 2011, which found a hazard ratio of 0.669 (95%CI: 0.54 to 0.828). Overall survival was 71.7% (95%CI: 67.8% to 75.7%) and 61.1% (95%CI: 56.8% to 65.3%) in the chemotherapy and observation groups, respectively. The 5-year relapse-free survival was 65.4% (95%CI: 61.2% to 69.5%) in the treatment arm compared to 53.1% (95%CI: 48.7% to 57.4%) in the surgery alone arm; hazard ratio was 0.653 (95%CI: 0.537 to 0.793). This reduction in hazard ratio was seen across all disease stages in subgroup analyses[37].

S-1, or tegafur, is not approved for use in the United States by the FDA. Based on pharmacokinetics studies, it has been documented that the drug is metabolized differently between Asians and Caucasians. The difference lies in the presence of CYP2A6, which occurs at a higher frequency in Eastern Asians. This enzyme is associated with reduced activity and subsequently reduced conversion of the prodrug *in vivo* to fluorouracil. Chuah *et al*[38] found that given the same dosing, the exposure to fluorouracil was similar in both ethnic groups. This was suggested by the investigators to be a result of increased renal clearance in Caucasians. Despite the same degree of exposure to the active metabolite, Caucasians were noted to have more grades 3 and 4 gastrointestinal toxicities compared to Asians (21% *vs* 0%)[38]. As a result of this difference, there is concern that tegafur use in the United States population may require dose reductions and efficacy of lower doses for resectable gastric cancer has not been addressed.

The First-Line Advanced Gastric Cancer Study evaluated an international cohort of patients with unresectable,

locally advanced or metastatic gastric and gastroesophageal adenocarcinoma using a protocol that compared S-1 and cisplatin with fluorouracil and cisplatin. It did not find significant differences in efficacy or toxicity profiles between the various ethnic groups[39]. This phase Ⅲ, randomized trial suggests that tegafur can be effective in Caucasians with advanced gastric cancer; however, further studies for resectable gastric carcinoma are warranted.

In 2012, a Korean group published results of the Capecitabine and Oxaliplatin Adjuvant Study in Stomach Cancer (CLASSIC) trial, which compared adjuvant capecitabine and oxaliplatin after D2 gastrectomy with R0 resection with surgery alone in stage Ⅱ and Ⅲ gastric adenocarcinomas. A total of 1035 patients were recruited between 2006 and 2009 in centers in South Korea, China, and Taiwan. Patients were randomized to either adjuvant chemotherapy or observation alone. Those assigned to chemotherapy received capecitabine (1000 mg/m^2 twice daily on days 1-14) and oxaliplatin (130 mg/m^2 on day 1) of a 3-wk cycle for a total of 8 cycles.

Median duration of follow-up was about 34 mo in both arms and 67% of those receiving chemotherapy completed 8 cycles of treatment. The 3-year disease-free survival was 74% (95%CI: 69% to 79%) and 59% (95%CI: 53% to 64%) in the chemotherapy and surgery alone groups, respectively, with a hazard ratio for chemotherapy of 0.56 (95%CI: 0.44 to 0.72, $P < 0.0001$). The 3-year overall survival was 83% (95%CI: 79% to 87%) in the treatment group compared to 78% (95%CI: 74% to 83%) in the observation group. The hazard ratio for overall survival was 0.72 (95%CI: 0.52 to 1.00, $P = 0.0493$). Estimation of median overall survival was not available at time of publication. In the subgroup analyses, survival benefit was seen in all disease stages and N1 and N2 diseases. There was no significant benefit for those with N0 disease[40].

A small randomized, double-blinded study was conducted to evaluate use of adjuvant FOLFOX4 *vs* fluorouracil/leucovorin in resectable gastric adenocarcinoma. A total of 80 patients were recruited from 2005 to 2009 after D2 gastrectomy with an R0 resection. Median duration of follow-up was about 36 mo. The 3-year overall survival was 36 mo in the FOLFOX4 group compared to 28 mo in the control group ($P < 0.05$). Similarly, the 3-year recurrence-free survival was 30 mo with the addition of oxaliplatin compared to 16 mo without ($P < 0.05$)[41].

Most recently, a phase Ⅲ study conducted by Kang *et al*[42] found an advantage using adjuvant cisplatin, mitomycin-C, and doxifluridine (iceMFP). Known as AMC 0101 trial, 521 patients were randomly assigned to receive mitomycin-C and doxifluridine (Mf, control) or the study arm, which included use of intraperitoneal cisplatin. The hazard ratio for recurrence in the iceMFP group was 0.70 (95%CI: 0.54 to 0.90, $P = 0.006$) with a 30% risk reduction for recurrence. The recurrence-free survival at 3 years was 60% (95%CI: 54% to 67%) in the study group compared to 50% (95%CI: 43% to 57%) in the control group. Median recurrence-free survival was not yet reached in the iceMFP arm but was 34.5 mo (95%CI: 24.2 to 63.8) in the Mf arm. Three-year overall survival rates were 71% (95%CI: 65% to 77%) and 60% (95%CI: 53% to 66%) for iceMFP and Mf, respectively[42]. Doxifluridine is another oral prodrug of 5-fluorouracil. Though doxifluridine is not FDA-approved for use in the United States, it is approved for use in Asia, calling into question the efficacy of cisplatin, mitomycin, and 5-fluorouracil (or its equivalent) in the United States.

ONGOING TRIALS AND FUTURE DIRECTIONS

Given the tenacious natural history of gastric cancer, many trials are currently ongoing to define more optimal treatments. Early phase I and Ⅱ data found promise in some new regimens, such as perioperative docetaxel, cisplatin, and capecitabine (DCX) and DCF[43,44], neoadjuvant S-1 and cisplatin or paclitaxel and cisplatin[45], and neoadjuvant docetaxel with S-1[46].

Of note, one highly anticipated trial, known as the Chemoradiotherapy after Induction Chemotherapy in Cancer of the Stomach trial, is a phase Ⅲ, randomized, multicenter trial designed to compare overall survival in patients with resectable gastric cancer when treated with 3 cycles of preoperative epirubicin, cisplatin, and capecitabine (ECC) followed by surgery and either an additional 3 cycles of ECC or concurrent chemoradiation with cisplatin, capecitabine, and 45 Gy. Accrual started in 2007 with results last updated in 2011, having enrolled 350 patients at that time[47].

In the United Kingdom, the MAGICB/ST03 study is exploring epirubicin, cisplatin and capecitabine (ECX) with or without bevacizumab followed by surgery, and adjuvant ECX with and without maintenance bevacizumab.

Neoadjuvant therapy is under study in a European trial comparing preoperative FU and cisplatin *vs* surgery alone and a joint Swiss/Italian trial of preoperative docetaxel, cisplatin and FU compared to surgery alone. Similarly, a Japanese study is evaluating preoperative cisplatin plus S-1 (an oral fluoropyrimidine) followed by surgery and postoperative S-1 *vs* surgery and postoperative S-1 alone (KYUH-UHA-GC04-03).

The Korean ARTIST Ⅱ trial is comparing adjuvant chemotherapy (S-1 *vs* S-1/oxaliplatin) with or without radiotherapy for completely resected gastric adenocarcinoma.

A randomized trial, the TOPGEAR trial, is underway in Europe and Canada to directly compare preoperative chemotherapy alone (ECF) *vs* chemoradiotherapy (two cycles of ECF followed by concurrent fluoropyrimidine-based chemoradiotherapy) in patients with resectable adenocarcinoma of the stomach and GEJ; both groups will receive three further cycles of ECF postoperatively

Uses of targeted agents are also being actively investigated. Recently, the REGARD trial, which was a random-

Table 1 Notable trial data for neoadjuvant and adjuvant therapies for gastric (or gastroesophageal) adenocarcinoma

Trial	No. of patients	Median survival (mo)	Overall survival	Progression-free survival
Neoadjuvant chemotherapy				
EORTC 40954[20]			(2 yr)	
5FU, cisplatin, folinic acid	72	64.62	72.70%	NR
Surgery alone	72	52.53	69.90%	NR
Perioperative chemotherapy				
MAGIC Trial[26]			(5 yr)	
ECF	250	NR	36.30%	NR
Surgery alone	253	NR	23%	NR
Fnlcc accord07/ffcd 9703[29]			(5 yr)	(5 yr)
5FU, cisplatin	113	NR	38%	34%
Surgery alone	111	NR	24%	19%
Adjuvant chemoradiation				
INT-0116 trial[32]			(3 yr)	(3 yr)
5FU, CRT	281	36	50%	48%
Surgery alone	275	27	41%	31%
Artist trial[34]				(3 yr)
Capecitabine, cisplatin, CRT	230	NR	NR	78.20%
Capecitabine, cisplatin	228	NR	NR	74.20%
Adjuvant chemotherapy				
ACTS-GC Trial[37]			(3, 5 yr)	(5 yr)
S-1	529	NR	80.1%, 71.7%	65.40%
Surgery alone	530	NR	70.1%, 61.1%	53.10%
Classic trial[40]			(3 yr)	(3 yr)
Capecitabine, oxaliplatin	520	NR	83%	74%
Surgery alone	515	NR	78%	59%

NR: Not reported; 5FU: 5-fluorouracil; ECF: Epirubicin/cisplatin/5-fluorouracil; CRT: Chemoradiation therapy.

ized, double-blinded, placebo-controlled, international study, established ramucirumab as an active biologic agent in advanced gastric cancer. Ramucirumab is a fully human IgG monoclonal antibody. It functions as a VEGFR-2 antagonist by preventing ligand binding and subsequent receptor-mediated pathway activation in endothelial cells, thus causing a decrease in tumor growth. Eligible patients had unresectable locally advanced recurrent or metastatic gastric or GEJ adenocarcinoma that progressed after first-line therapy. The majority population in both arms (approximately 75%) were patients with gastric adenocarcinoma. Median overall survival was 5.2 mo with ramucirumab and 3.8 mo with placebo. Hazard ratio was 0.776 (95%CI: 0.603 to 0.998, $P = 0.047$). Estimated overall survival and progression free survival were also improved[48]. This pivotal study established the role of ramucirumab as a single agent in advanced or metastatic gastric cancer. Further studies are sure to follow.

In the United Kingdom, the MAGICB/ST03 study is exploring epirubicin, cisplatin and capecitabine (ECX) with or without bevacizumab followed by surgery, and adjuvant ECX with and without maintenance bevacizumab.

The ToGA trial established use of trastuzumab in HER2-positive metastatic gastric cancer[49]. Similar promise was found with the use of trastuzumab in combination with chemotherapy[50-53] and additional clinical trials are currently underway. For instance, the TOXAG study is a phase Ⅱ clinical trial looking at the safety profile of adjuvant oxaliplatin, capecitabine, and trastuzumab with radiation. It is currently recruiting patients.

With respect to surgical interventions, new modes of treatment are being reviewed. A randomized trial known as CCOG 1102 has been planned to study the efficacy of extensive intraoperative peritoneal lavage compared to traditional surgery in resectable advanced gastric cancer with a primary end point of disease-free survival. A total of 300 patients are planned for accrual[54]. And finally, in regards to the controversy surrounding the extent of lymphadenectomy, a prospective randomized trial has been planned to compare D1 and D2 lymphadenectomy with a primary endpoint of 5-year overall survival.

CONCLUSION

Adenocarcinoma of the stomach, unfortunately, carries a poor prognosis and has a high mortality rate despite current available therapies. Most clinicians now treat GEJ and proximal gastric (*i.e.*, cardia) cancers as esophageal cancers, using preoperative chemoradiotherapy. However, it is important to note that tumors arising from within 5 cm of the GEJ without extension into the esophagus are classified in the same category as gastric cancer according to the updated AJCC Staging Manual and should be treated as such. This review outlines evidence-based approaches in the management of this difficult disease.

For patients with non-cardia gastric cancer, randomized trials and meta-analyses provide support for a number of approaches including adjuvant chemoradiotherapy, as shown in the INT-0116 trial, perioperative chemotherapy (preoperative plus postoperative), as was used in the MAGIC trial. Few studies have compared these approaches; however, the optimal way to integrate

combined modality therapy has not been definitively established. Decisions are often made based on institutional and/or patient preference. A major problem, at least in the United States, is that some patients with gastric cancer undergo surgery prior to consultation by medical or radiation oncologists.

Currently, a multidisciplinary approach and definitive surgical resection are recommended for locally advanced, early stage cancer. The gastrectomy should be performed laparoscopically if possible. It should be with negative margins and accompanied by a D1 lymphadenectomy with at least 15 lymph nodes sampled. A D2 lymphadenectomy should be performed in well-experienced centers.

For patients who have already undergone potentially curative gastric resection, we suggest adjuvant chemoradiotherapy rather than surgery alone for patients with N1 disease (which would include T1N1 stage IB), and for patients with T3N0 (stage ⅡA) disease and above, based upon the results of US Intergroup trial INT-0116[22]. For the subgroup of patients with T2N0 disease, either observation or adjuvant treatment is acceptable, and the decision can be based upon individualized patient (such as age, performance status, and motivation for treatment) and disease risk factor (*e.g.*, histologic grade or the presence of lymphovascular or perineural invasion) considerations.

An acceptable alternative approach for patients who are seen prior to resection is perioperative chemotherapy alone (ECF). It is reasonable to select patients utilizing the eligibility criteria for the MAGIC trial (patients of any age with a performance status of 0 or 1), a histologically proven adenocarcinoma of the stomach that was considered to invade through the submucosa (stage T2 or higher), with no evidence of distant metastases or locally advanced inoperable disease, as evaluated by CT, ultrasonography or laparoscopy[17].

East Asian patients with resected node-positive disease or T3N0 (stage ⅡA) disease and above, may take one year of postoperative S-1 chemotherapy. It is difficult to know whether the benefit of adjuvant therapy with S-1, as demonstrated in the Japanese ACTS-GC trial[26], can be extrapolated to other populations, given the markedly better outcomes seen in both the treated and the surgery alone control groups, stage for stage, when compared to outcomes in other non-Japanese populations. Until further information becomes available, we suggest that this approach be limited to East Asian patients. Other alternative chemotherapy regimens for adjuvant therapy include capecitabine plus oxaliplatin, as was used in the CLASSIC trial[29], or capecitabine plus cisplatin, as was used in the ARTIST trial[24]. Table 1 summarizes the available data from pivotal trials.

As technology moves increasingly toward molecular targeted therapy, biologic agents such as trastuzumab and ramucirumab hold great promise in the treatment of this disease as well. Their roles have not yet been defined in locally advanced gastric cancer but they are important new advances in the era of personalized medicine.

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MINIREVIEWS

Neoadjuvant therapy for esophageal cancer

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Abstract

Esophageal cancer is increasing in incidence more than any other visceral malignancy in North America. Adenocarcinoma has become the most common cell type. Surgery remains the primary treatment modality for locoregional disease. Overall survival with surgery alone has been dismal, with metastatic disease the primary mode of treatment failure after an R0 surgical resection. Cure rates with chemotherapy or radiation therapy alone have been disappointing as well. For these reasons, over the last decade multi-modality treatment has gained increasing acceptance as the standard of care. This review examines the present data and role of neoadjuvant treatment using chemotherapy and radiation therapy followed by surgery for the treatment of esophageal cancer.

Key words: Neoadjuvant therapy; Esophageal cancer; Esophagectomy; Chemotherapy

Core tip: This review evaluates the current literature on the use of neoadjuvant chemotherapy with or without radiation therapy for the treatment of locally advanced esophageal cancer. Major randomized controlled trials and co-operative group studies have been evaluated. Response rates, survival, complete response and outcomes have been thoroughly reviewed.

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INTRODUCTION

Nearly 500000 patients are diagnosed with esophageal cancer worldwide yearly, and its incidence has nearly doubled in North America over the last 2 decades[1]. Adenocarcinoma is now the most common cell type in the western hemisphere followed by squamous cell cancer[2]. For locoregional disease, surgery has been the mainstay of therapy with 5-year survival rates ranging from 10%-40% and distant metastasis being the most common mode of treatment failure[3]. Radiation therapy alone has been evaluated for local control and, in one large series 3-year survival was only 6%[4]. Chemotherapy for locally advanced esophageal cancer has a response rate of 45% to 75% in numerous studies but relapse rates are high and long-term survival rates are very low.

Use of chemotherapy with or without radiation therapy before surgery has several theoretical benefits. It may improve baseline dysphagia, the most common symptom on presentation. It can help downstage the tumor, which may increase resection rates, and can treat micro-metastatic disease that is not detected on imaging studies. It has the potential to indicate the biologic behavior of the tumor by its response to treatment that may help guide further therapy.

The role of multi-modality treatment as a way to achieve higher long-term survival rates has been debated for many years. The roles of chemotherapy and radiation therapy to improve surgical results remain controversial; randomized trials have shown mixed results. This review will examine the data and survival rates for using pre-

operative chemotherapy and radiation therapy, alone or in combination, in the management of localized esophageal cancer.

NEOADJUVANT CHEMOTHERAPY

In a study by Boonstra *et al*[5], 169 patients with squamous cell cancer were randomized to 2-4 cycles of cisplatin and etoposide followed by surgery or surgery alone. Median overall survival in the two groups was 16 and 12 mo respectively. The 5-year survival in the chemotherapy group was 26% *vs* 17% in the surgery alone group ($P = 0.03$, hazard ratio 0.71; 95%CI: 0.51-0.98). Contrary to this study result, a large North American Intergroup 113 trial failed to show a survival benefit for three cycles of preoperative cisplatin/5-FU followed by surgery and two additional cycles of cisplatin/5-FU compared to surgery alone[3]. Both squamous and adenocarcinoma patients were included. With a study size of 440 patients, overall survival in each group was 20% and there was no benefit of chemotherapy seen in resection rates, local failure, or distant metastasis.

In a much larger study by the Medical Research Council Oesophageal Cancer Working group[6], 802 patients were randomized to two cycles of cisplatin/5-FU followed by surgery *vs* surgery alone. Median and 2-year survivals were improved in the chemotherapy group (16.8 mo *vs* 13.3 mo-difference 107 d; 95%CI: 30-196, and 43% *vs* 34%-difference 9%; 95%CI: 3-14, respectively). The curative resection rate was improved marginally from 55% to 60%. The MAGIC trial, performed in the United Kingdom[7], further reinforced the findings seen in the Medical Research Council study. A total of 503 patients with distal esophageal, GE junction and gastric adenocarcinoma were randomized to three cycles of pre and postoperative cisplatin/5-FU/epirubicin or surgery alone. Overall survival in the chemotherapy group was significantly better (36% *vs* 23%, $P = 0.009$), but fewer than one third of the patients in this study had distal esophageal adenocarcinoma. In a French study[8] of 224 patients randomized to 2-3 cycles of preoperative cisplatin/5-FU followed by surgery *vs* surgery alone, there was a significantly improved R0 resection rate (84% *vs* 73%, $P = 0.04$), 5-year disease free survival (34% *vs* 21%, $P = 0.003$), and 5-year overall survival (38% *vs* 24%, $P = 0.02$) following chemotherapy.

The data published in these studies are quite heterogeneous. Some studies have both squamous and adenocarcinoma patients while some have only adenocarcinoma patients. The chemotherapy drugs and regimens vary between studies as well. In a meta-analysis of 12 randomized trials in which pre-operative chemotherapy was used, the 5-year overall survival benefit was only 4%[9]. The benefit was somewhat smaller for squamous cell cancer compared to adenocarcinoma (4% *vs* 7%). Thus, the available data do not suggest that the use of neoadjuvant chemotherapy significantly improves survival.

NEOADJUVANT RADIATION THERAPY

In a trial of 96 patients by Kelsen *et al*[10], patients were assigned to preoperative radiotherapy or chemotherapy. The morbidity and mortality of surgery following preoperative treatment was no different compared to historical controls of surgery alone but there was no survival benefit of preoperative treatment. Another randomized trial of 176 patients comparing preoperative radiation (20 Gy in 10 treatments) followed by surgery *vs* surgery alone[11] showed no benefit of radiotherapy with overall 5-year survival of 13%. In a Scandinavian trial of 186 patients, Nygaard *et al*[12] showed an improved 3-year survival in patients receiving preoperative radiotherapy compared to patients undergoing surgery alone or chemotherapy and surgery.

A meta-analysis has not shown a statistically significant survival benefit for preoperative radiation[13]. At a median follow-up of 9 years, the survival benefit at 2 and 5 years was 3% and 4% respectively ($P = 0.062$). Thus neoadjuvant radiation therapy alone cannot be advocated for the management of esophageal cancer.

NEOADJUVANT CHEMORADIOTHERAPY (COMBINED THERAPY, TRIMODALITY THERAPY)

Neither preoperative radiation therapy nor chemotherapy alone in the neoadjuvant setting have been proven beneficial based on the trials[5,7-9] performed. This may be related to the low complete pathologic response rates, mostly between 2.5%-4%. The improvement in R0 resection and overall survival has been limited as well. Most patients who undergo surgical resection die from distant metastatic disease in spite of an R0 resection. Considering these results and for the reasons listed earlier in this review for using neoadjuvant therapy, combination therapy with all three modalities has been utilized to try to improve overall outcomes. We will first review the studies looking at trimodality therapy vs surgery alone.

Trimodality therapy vs surgery alone

Bosset *et al*[14] randomized 282 patients to preoperative cisplatin and concurrent radiation or surgery alone. Although the curative resection rate was higher with combined therapy (81% *vs* 69%), disease-free survival was improved (HR 0.6, 95%CI: -0.4-0.9, $P = 0.003$), and risk of local recurrence decreased (HR 0.6, 95%CI: -0.4-0.9, $P = 0.01$), there was no difference in overall survival. This may at least in part be due to higher than expected treatment related mortality in the chemo-radiation arm (12% *vs* 4%). This study only included patients with squamous cell cancers, and radiation was given using a split-dose technique.

Burmeister *et al*[15] in an Australian study randomized 256 patients to one cycle of cisplatin/5-FU and radiation

followed by surgery or to surgery alone. R0 resection was achieved in 80% of the patients in the combined therapy group *vs* 59% in the surgery alone arm. However, the overall survival was no different between the two groups. Patients with adenocarcinoma had a decreased rate of complete pathologic response and were more likely to have disease progression during the follow-up period.

In a study from the University of Michigan[16], 100 patients were randomized to preoperative cisplatin/5-FU/vinblastine plus radiation or to surgery alone. There was a significant decrease in the rate of local recurrence with combined therapy (19% *vs* 42%, $P = 0.03$) and a trend towards improved survival at 3 years (30% *vs* 16%, $P = 0.15$). In an Irish study of 113 patients, Walsh *et al*[17] showed a significant improvement in overall survival at 3 years (32% *vs* 6%) with preoperative cisplatin/5-FU/radiation followed by surgery *vs* surgery alone. All patients in this study had adenocarcinoma but the extremely poor 3-year survival in the surgery alone arm (6%) could not be explained. In 2012, a multi-institutional phase Ⅲ study (CROSS trial)[18] evaluated the benefit of induction therapy using carboplatin/taxol/41Gy radiation *vs* surgery alone. Only a quarter of the patients had squamous histology. There was an anastomotic leak rate of 22%-30% in each arm. Median survival was 49 mo in the combined therapy arm compared to 24 mo in the surgery arm ($P = 0.003$). The overall 5-year survival was much improved in the combined therapy arm (47% *vs* 34%, $P = 0.03$). Patients with squamous histology derived a larger benefit. An updated analysis[19] of this group of patients showed a lower local recurrence rate (34% *vs* 14%, $P < 0.001$) and lower risk of peritoneal carcinomatosis (14% *vs* 4%, $P < 0.001$) following neoadjuvant chemoradiation and that squamous cell carcinoma was an independent prognostic variable in the surgery alone group.

Neoadjuvant chemoradiation vs neoadjuvant chemotherapy alone

Stahl *et al*[20] reported their data of 120 patients with T3 or higher and/or node positive patients who were randomized to preoperative cisplatin/5-FU/leucovorin followed by surgery *vs* cisplatin/5-FU/leucovorin followed by chemoradiotherapy with cisplatin/etoposide and then surgery. Trimodality patients had a higher rate of pathologic complete response (16% *vs* 2%, $P = 0.03$) and node-negative status (64% *vs* 37%, $P = 0.01$). The overall 3-year survival was not statistically significantly different in the two groups with a median overall survival of 32.8 *vs* 21.1 mo ($P = 0.14$).

In a recent meta-analysis[9] of 10 randomized trials of trimodality therapy *vs* surgery alone and 8 trials of preoperative chemotherapy *vs* surgery alone, trimodality therapy was associated with a 13% benefit in survival at 2 years, both in squamous and adenocarcinoma. Preoperative chemotherapy alone translated to a 7% benefit in survival at 3 years, more in adenocarcinoma than in squamous cell cancer. Thus, these data suggest a synergistic benefit using neoadjuvant chemotherapy plus radiotherapy in the management of esophageal cancer.

CONCLUSION

The three mainstays of treatment for esophageal cancer-surgery, chemotherapy, and radiation therapy result in poor overall survival and high relapse rates when used alone. Preoperative combination therapy offers several theoretical advantages but for stage 1 and 2 esophageal cancers, there is, as of now, no convincing evidence that neoadjuvant chemoradiation is of any benefit. Neoadjuvant chemoradiotherapy achieves the highest complete pathologic response rates, R0 resection rates, and improves 3-5 years survival rates in patients with locally advanced esophageal cancer. The addition of neoadjuvant radiotherapy to preoperative chemotherapy may facilitate a better complete surgical resection *via* its effect on the periphery of the tumor. Squamous cell cancer and adenocarcinoma appear to have similar disease-free and overall survival rates following neoadjuvant chemoradiotherapy. Further randomized, prospective trials will be required to build on these early studies to try to improve the prognosis of patients with this terrible disease.

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MINIREVIEWS

Peritoneal metastases of colorectal origin treated by cytoreduction and HIPEC: An overview

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Abstract

Colorectal peritoneal carcinomatosis was considered a terminal condition with a merely palliative treatment that included only supportive care, palliative surgery and the best systemic chemotherapy. Since the birth of a new approach, cytoreductive surgery with peritonectomy procedures together with hyperthermic intraperitoneal chemotherapy and/or early postoperative intraperitoneal chemotherapy to treat peritoneal carcinomatosis, many research groups contributed with promising results using this procedure being up to date this strategy the only one that has shown curative benefits on colorectal peritoneal carcinomatosis achieving reported overall survival rates up to 64 mo and five-year survival rates up to 51%. The aim of this paper is to expose an updated overview of the therapeutic possibilities of these procedures in colorectal peritoneal metastases in the same way that our Unit of Oncologic Surgery has performed since 1997 with more than four hundred procedures.

Key words: Carcinomatosis peritoneal; Colon cancer; Intraperitoneal chemotherapy; Cytoreduction; Peritonectomy

Core tip: The carcinomatosis peritoneal from colon origin has turned from a terminal condition to a curative scenery. The cytoreduction and peritonectomy procedures with hyperthermic intraperitoneal chemotherapy have achieved 50% in 5 years overall survival, with a low morbidity that is not higher than other major surgical procedures.

Arjona-Sánchez A, Medina-Fernández FJ, Muñoz-Casares FC, Casado-Adam A, Sánchez-Hidalgo JM, Rufián-Peña S. Peritoneal metastases of colorectal origin treated by cytoreduction and HIPEC: An overview. *World J Gastrointest Oncol* 2014; 6(10): 407-412 Available from: URL: http://www.wjgnet.com/1948-5204/full/v6/i10/407.htm DOI: http://dx.doi.org/10.4251/wjgo.v6.i10.407

INTRODUCTION

Colorectal cancer (CRC) is considered the third most common cancer. One of the major aspects related to treatment failure is the appearance of peritoneal metastases (PM), which are thought to be present in about 40% of patients with CRC at some time during the natural history of this disease[1]. The occurrence of PM may be a result of the growth of the primary tumor allowing the exfoliation of malignant cells intraperitoneally when the serosa is exceeded or be the consequence of a surgical manipulation when lymphatics or blood vessels are transected.

In the past, colorectal peritoneal carcinomatosis was considered a terminal condition with a merely palliative treatment that included only supportive care, palliative surgery and the best systemic chemotherapy, achieving

survival rates not exceeding seven months according to the multicenter study EVOCAPE[2] with 5-FU and Leucovorin, reaching up to 23.4 mo survival with modern chemotherapy like Oxaliplatine and Irinotecan[3]. Fortunately, in the 80's decade, a renewed interest in malignant diseases with peritoneal extension and the introduction of the concept of initial loco-regional disease resulted in the birth of a new approach. Thus, Elias *et al*[4] described and popularized several procedures, including cytoreductive surgery (CRS) (with peritonectomy procedures) together with hyperthermic intraperitoneal chemotherapy (HIPEC) and early postoperative intraperitoneal chemotherapy (EPIC), to treat peritoneal carcinomatosis[5]. Many research groups contributed with promising results using complete cytoreduction of macroscopic disease combined with HIPEC in order to treat microscopic disease. Although preliminary data were viewed with great scepticism, to date, this strategy is the only one that has shown curative benefits on colorectal peritoneal carcinomatosis achieving reported overall survival rates up to 46 mo[6] and five-year survival rates up to 51%[3].

The aim of this paper is to expose an updated overview of the therapeutic possibilities of these procedures in colorectal PM.

PATIENT SELECTION

The importance of a good general health status must be emphasized. The candidates for these procedures should be younger than 70 years with physiological age of less than 65 years, but it is a relative condition. Severe cardiorespiratory disease, renal failure, untreated malignant neoplasm or World Health Organization (WHO) index > 2 are considered major contraindications to CRS + HIPEC[7]. Furthermore, all patients included to CRS with curative intention shouldn't present tumour progression while on chemotherapy. The key to a successful outcome is an appropriate selection of patients in order to achieve complete cytoreduction, since this is an essential prognostic factor[8]. To this respect, it has been demonstrated that patients with incomplete cytoreduction and residual tumor ⩾ 2.5 mm don't achieve more than 6 mo survival[9,10].

In that sense, preoperative evaluation should include complete colonoscopy and CT scan of the chest and abdomen, focused the attention on radiologic manifestations of PM such as: ascites, peritoneal nodules or masses, peritoneal thickening and enhancement or mesenteric effacement. In those cases in which any extra-peritoneal or extra-abdominal disease is suspected, positron emission tomography (PET) may be useful to evaluate the extension of the disease.

From a preoperative point of view, some authors have related certain preoperative clinical and radiological variables with the possibility of achieving complete cytoreduction. Among them, it is worth to remark, the absence of extra-abdominal disease, not more than 3 small-size and resectable liver metastases, no high volume of disease in the gastrohepatic ligament, no evidence of multiple enteric, ureteric or biliary obstruction, as well as no evidence of gross involvement of mesentery or several segments of intestine which cause intestinal obstruction[11].

The extension of the peritoneal disease represents one of the major prognosis factors for survival and, thus, could represent another criteria for patient selection. To quantify it, several index have been proposed, but presently, the most widely used is the Peritoneal Cancer Index (PCI) described by Sugarbaker. In relation to this index, some authors have considered that a PCI higher than 10 lead to a worse prognosis and a score greater than 20 as a possible contraindication to CRS and HIPEC, as the 5-year survival rate in patients with PCI > 19 is 7%[10]. To evaluate more accurately PCI, diagnostic laparoscopy may be useful as reported by Valle *et al*[12] who performed staging laparoscopy in 97 patients, achieving good correlation between the PCI subsequently assessed at the time of laparotomy. However, this is a challenging evaluation procedure, especially in those patients previously operated on, due to the risk of iatrogenic injury during the exploration.

In addition to the PCI, recently, a new preoperative severity index of peritoneal carcinomatosis called "Peritoneal Surface Disease Severity Score" (PSDSS) has been described. This score, which includes the PCI and other variables such as clinical symptomatology and histopathology of the primary tumor, consists on four grades, showing that the stages III and IV have a negative impact on survival (Table 1)[13].

The presence of multiple liver metastases represents a relative contraindication as several studies have shown that there is no negative impact on survival rates when liver metastases are inferior to 3, chemo-sensitive, and can be fully resected at the time of surgery[14]. In this study, 3 year-overall and disease-free survivals were 41.5% and 26% respectively. In the same line, other authors have observed similar findings in similar scenarios, especially when PCI is low[15]. On the contrary, the presence of extra-abdominal metastases and massive retroperitoneal lymphatic involvement, mainly in cases of non-responsive to systemic chemotherapy, should be considered absolute contraindications. Nevertheless, some authors have proposed that extrahepatic disease might not be a contraindication to attempt an R-0 resection if the number of sites of metastases is less than five[16].

CYTOREDUCTIVE SURGERY WITH PERITONECTOMY AND PERIOPERATIVE INTRAPERITONEAL CHEMOTHERAPY PROCEDURES

Maximum CRS aims to remove all macroscopic disease using extensive visceral resections and peritonectomy procedures as described by Sugarbaker[5]. When tumour fully invades the visceral surface of different organs,

Table 1 Peritoneal Surface Disease Severity Score

Symptomatology	PCI	Histology
No symptoms (0)	< 10 (1)	Well differentiated or moderately differentiated + N0 (1)
Moderate symptoms (1)	10-20 (3)	Moderately differentiated + N1 or N2 (3)
Severe symptoms (6)	> 20 (7)	Poorly differentiated or ring seal (9)

(): Score. Moderate symptoms is defined as weight loss of < 10%, moderate abdominal pain, ascites asymptomatic. Severe symptomatology is defined as weight loss of > 10%, pain that continues, intestinal obstruction, symptomatic ascites. PCI: Peritoneal Cancer Index (0-39). Histology of the primary tumor. N regional lymph node metastasis. Grade Ⅰ: Summation result = (2-3); Grade Ⅱ: (4-7); Grade Ⅲ: 8-10; Grade Ⅳ: > 10.

resection may be necessary. One of the major technical limitations found by an oncological surgeon is the whole involvement of the small bowel as prevents to perform a complete tumour cytoreduction.

The realization of CRS along with HIPEC improves the outcomes in a single surgical act. However, to achieve this goal, an optimal debulking without macroscopic tumor residue (CC-0 resection) or with a tumor residue less than 2.5 mm (CC-1 resection) must be accomplished, since complete cytoreduction has been shown the most important prognostic factor for survival[17,18]. Other major prognostic factors associated with worse outcomes are: grades 2 and 3 *vs* grade 1 histopathologic grade, PCI > 20, lymph node-positive primary tumors and volume of preoperative PM[17-19].

Intuitively, minimally invasive approach for therapeutic purpose might appear not to be useful in this setting, nevertheless, in carefully selected patients, totally laparoscopic CRS and HIPEC has been performed successfully. In that way, Esquivel *et al*[20] have reported success rates up to 95% with acceptable morbidity in patients with a PCI < 10[21,22]. Although others authors have also remarked this possibility, these data are preliminary and must be taken cautiously.

Intraperitoneal administration of cytostatic drugs presents pharmacokinetic advantages because of the plasma-peritoneum barrier that allows the administration of loco-regional high doses of chemotherapy with minimal systemic effects. This characteristic may also lead to a positive effect on recurrence and survival rates[4]. Perioperative administration lead to an extensive intrabdominal diffusion without any of limitations related to postoperative adhesions. Furthermore, hyperthermia has shown greater cytotoxic capacity. Therefore, in *in vitro* tests at 42.5 °C, certain cytostatic drugs such as Oxaliplatin, Mitomycin C, Doxorubicin, Irinotecan or Cisplatin, have demonstrated to increase their cytotoxicity and penetration, and thus, their antitumor effects[23]. However, at present, the use of HIPEC is only indicated in cases achieving complete cytoreduction since the penetration of intraperitoneal chemotherapy is limited to several millimetres. On the other side, the administration of EPIC is related to a higher morbidity as Elias *et al*[24] showed in randomized trial as the use of this variety of chemotherapy has been introduced in different treatment protocols[25].

New chemotherapy drugs such as bevacizumab, an humanized monoclonal antibody that produces angiogenesis inhibition by inhibiting vascular endothelial growth factor A (VEGF-A), are being tested at the moment in animal models and might be useful as perioperative chemotherapeutic agent in the next future[26,27].

SURVIVAL OUTCOMES AND MORBIMORTALITY OF CYTOREDUCTIVE SURGERY AND HIPEC

The results contributed by many authors, although mainly in a retrospective way, demonstrate that degree of cytoreduction is the most determining factor for survival. All comparative trials report a median survival superior to 2 years for patients treated with complete CRS (CC-0) or with residual tumor less than 2.5 mm (CC-1), reaching some of them survival rates above 50% at 5 years[3,28]. Dutch randomized phase III trial conducted by Verwaal *et al*[9,29] first published in 2003 and latest updated in 2008, compared CRS and HIPEC (Mitomycin C) with intravenous chemotherapy and palliative surgery as sole treatment in patients suffering from colorectal peritoneal carcinomatosis. This trial showed significant differences in terms of overall survival (22.2 mo *vs* 12.6 mo), and a 5-year survival up to 45% in favour of the patients treated with CRS and HIPEC. These data forced to stop the trial for ethical issues. In addition, another similar study conducted by Elias *et al*[3] that compared latest systemic chemotherapy to CRS and HIPEC showed a significantly better outcomes in favour of the combined procedure, reaching a median survival of 63 mo and 51% at 5 years overall survival, being these, the best outcomes reported to date using CRS and HIPEC in colorectal PM.

To date, only one systematic review and meta-analysis has been published regarding CRS + HIPEC in colorectal PM. In that study, de Cuba *et al*[30] concluded that when liver metastases are presented in addition to isolated PM, there is a trend towards a lower overall survival after curative resection. Furthermore, these authors also support that CRS + HIPEC is superior to modern systemic chemotherapy in increasing overall survival.

Since 2003, numerous studies reporting the outcomes of CRS and HIPEC have been published. Table 2 summarizes the characteristics of most of them.

On the other hand, since CRS and HIPEC were described, these procedures have been criticized due to a high morbidity. This fact could be true at the beginning; however, currently the morbidity, when this surgery is performed in experienced units, is not superior to that which presents any major gastrointestinal surgery. In that sense, the combination of CRS and HIPEC is a complex procedure that exposes the patient to an acceptable mor-

Table 2 Survival outcomes of patients underwent cytoreductive surgery and hyperthermic intraperitoneal chemotherapy

Ref.	Type of study	Year	*n*	Overall survival (mo)	Five-year survival	Overall morbidity[1]	Perioperative mortality
Verwaal *et al*[9]	RCT	2003	39	22	NR	NR	NR
Glehen *et al*[31]	RMS	2004	377	32	40%	22.9%	4%
da Silva *et al*[17]	RS	2006	70	33	32%	NR	NR
Kianmanesh *et al*[15]	RS	2007	30	38	44%	39%	2.3%
Bijelic *et al*[32]	RS	2008	49	33	20%	NR	NR
Shen *et al*[33]	RS	2008	121	34	26%	42%	5.5%
Yan *et al*[34]	RS	2008	50	29	NR	NR	NR
Elias *et al*[3]	CRS	2009	48	63	51%	NR	NR
Chua *et al*[19]	RS	2009	54	33	NR	NR	NR
Franko *et al*[28]	CRS	2010	67	34.7	26%	NR	NR
Elias *et al*[10]	RMS	2010	523	32	30%	31%	3%
Quenet *et al*[35]	PS	2011	146	41	41.8%	47.2%	4.1%
Ung *et al*[6]	RS	2013	211	46.8	42%	NR	NR

[1]Morbidity data comes from different classifications and grades, so major morbidity might be lower in most cases. RCT: Randomized clinical trial; RMS: Retrospective multicenter study; RS: Retrospective Study; CRS: Comparative Retrospective Study; PS: Prospective Study; NR: Not reported.

bidity and mortality (Table 2). To this respect, main high-grade morbidity of these patients is related to surgery and presented in form of anastomotic leak, intraperitoneal sepsis or abscesses, and hematologic and renal toxicities related with HIPEC. Multivariate analyses including in different studies show the extension of disease, number of anastomosis, duration of intervention and incomplete cytoreductive surgery as independent risk factors for morbidity[10].

RECOMMENDATIONS FOR THE MANAGEMENT OF PATIENTS DIAGNOSED FOR COLORECTAL PERITONEAL CARCINOMATOSIS

All surgeons or oncologists diagnosing a colorectal peritoneal carcinomatosis, before, during or after surgery; especially in young patients with limited disease, should consider the evaluation of the case for a multidisciplinary team in a specialized unit in order to offer the realization of this therapeutic approach with curative intent. An exploratory laparotomy without a description of the extent of the disease should be a prohibited action. In this sense, when a peritoneal carcinomatosis is discovered intraoperatively, it is recommended that the surgeon describe in detail the extension and allocation of PM according to the PCI. This conduct will allow the correct evaluation of these patients in specialized units, avoiding inappropriate transfers, resource consumptions and discomfort to the patient. Likewise, a very detailed description of the PM extent will prevent an unnecessary laparotomy in those cases in which a complete cytoreduction is not possible[11].

In the same way, the realization of CRS without HIPEC should be avoided since this conduct limits the possibility of receiving a combined treatment with curative intent and better outcome. Resection of peritoneum without HIPEC allows free tumor cells to implant and grow all over the abdominal cavity, which impairs future treatment options and increase the risk of morbidity[11]. From this point of view, there are a group of patients that although undergoing complete resection without HIPEC, are at high-risk of developing colorectal peritoneal carcinomatosis. Thus, resected minimal synchronous macroscopic PM, synchronous ovarian metastases and perforated primary tumors could benefit of second-look surgery with CRS and HIPEC as it seems to be that up to 55% of asymptomatic patients may present PM at one year[36].

Finally, an emergency surgeon that incidentally is faced with a colorectal peritoneal carcinomatosis should avoid unnecessary surgical dissection and solve the urgent situation (obstruction and/or perforation and/or abdominal sepsis) using the minimum necessary surgical gesture.

CONCLUSION

At present, CRS and HIPEC procedures represent a therapy with curative intent in selected patients with colorectal peritoneal carcinomatosis. The finding of a peritoneal carcinomatosis requires surgeons and oncologists to not ignore this treatment option and to refer such patients to experienced units in the treatment of peritoneal surface malignancies, in order to limit morbidity and increase their survival.

It is clear that there are many unknowns pending to be solved in the next few years such as different modes, time, dose, temperature and drugs for HIPEC to decrease local recurrence after CC-0 resections. Furthermore, at this moment, several trials are evaluating the role of second-look surgery with CRS + HIPEC as well as the possibility of prophylactic HIPEC when primary colorectal cancer shows synchronous PM or is a high risk patient to develop carcinomatosis[36]. These novel strategies might be incorporated in the future therapeutic protocols of colorectal PM.

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RETROSPECTIVE STUDY

Plasma monocyte chemotactic protein-1 remains elevated after minimally invasive colorectal cancer resection

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Abstract

AIM: To investigate plasma Monocyte Chemotactic Protein-1 levels preoperatively in colorectal cancer (CRC) and benign patients and postoperatively after CRC resection.

METHODS: A plasma bank was screened for minimally invasive colorectal cancer resection (MICR) for CRC and benign disease (BEN) patients for whom preoperative, early postoperative, and 1 or more late postoperative samples (postoperative day 7-27) were available. Monocyte chemotactic protein-1 (MCP-1) levels (pg/mL) were determined *via* enzyme linked immuno-absorbent assay.

RESULTS: One hundred and two CRC and 86 BEN patients were studied. The CRC patient's median preoperative MCP-1 level (283.1, CI: 256.0, 294.3) was higher than the BEN group level (227.5, CI: 200.2, 245.2; P = 0.0004). *Vs* CRC preoperative levels, elevated MCP-1 plasma levels were found on postoperative day 1 (446.3, CI: 418.0, 520.1), postoperative day 3 (342.7, CI: 320.4, 377.4), postoperative day 7-13 (326.5, CI: 299.4, 354.1), postoperative day 14-20 (361.6, CI: 287.8, 407.9), and postoperative day 21-27 (318.1, CI: 287.2, 371.6; $P < 0.001$ for all).

CONCLUSION: Preoperative MCP-1 levels were higher in CRC patients (*vs* BEN). After MICR for CRC, MCP-1 levels were elevated for 1 mo and may promote angiogenesis, cancer recurrence and metastasis.

Key words: Colorectal cancer; Monocyte chemotactic protein-1; Minimally invasive colorectal resection angiogenesis

Core tip: In our past published studied we have shown that plasma levels of the pro-angiogenic proteins, vascular endothelial growth factor, angiopoietin-2, placental growth factor, and soluble vascular adhesion

molecule-1, are significantly elevated for 2-4 wk following minimally invasive colorectal resection for colorectal cancer (CRC). Additionally, we also showed that postoperative plasma from cancer patients stimulates *in vitro* endothelial cell proliferation, migration, and invasion, all of which are critical steps in angiogenesis. In this manuscript we are presenting data to show that plasma Monocyte chemotactic protein-1 (MCP-1), a pro-angiogenic protein, in CRC patients remain elevated for month after MICR. Furthermore, we are also showing that the median preoperative plasma level of MCP-1 is significantly higher in the CRC patients than in the BEN group.

Shantha Kumara HMC, Myers EA, Herath SAC, Jang JH, Njoh L, Yan X, Kirchoff D, Cekic V, Luchtefeld M, Whelan RL. Plasma monocyte chemotactic protein-1 remains elevated after minimally invasive colorectal cancer resection. *World J Gastrointest Oncol* 2014; 6(10): 413-419 Available from: URL: http://www.wjgnet.com/1948-5204/full/v6/i10/413.htm DOI: http://dx.doi.org/10.4251/wjgo.v6.i10.413

INTRODUCTION

Surgery remains the mainstay of treatment for colorectal cancer (CRC), however, a significant number of patients develop disease recurrence following a "curative resection", presumably from unrecognized tumor microfoci or from viable tumor cells that persist in the circulation[1]. There is growing evidence that tumor resection may indirectly stimulate the growth of residual cancer *via* surgery-related immunosuppression and elevated blood levels of proangiogenic proteins during the early postoperative period. Thus, the early postoperative period may be a dangerous time window for cancer patients who potentially harbor residual disease.

Plasma levels of the proangiogenic proteins, vascular endothelial growth factor (VEGF), angiopoietin-2 (Ang-2), placental growth factor (PlGF), and soluble vascular adhesion molecule-1 (sVCAM-1), have been noted to be significantly elevated for 2-4 wk following minimally invasive colorectal resection for CRC[2-5]. Additionally, prior studies have shown that postoperative plasma from cancer patients stimulates *in vitro* endothelial cell (EC) proliferation, migration, and invasion, all of which are critical steps in angiogenesis[6].

Monocyte chemotactic protein-1 (MCP-1), a member of the C-C chemokine family, is a protein that has several proangiogenic effects. Evidence shows that MCP-1 is produced by certain tumor cells as well as stromal cells such as fibroblasts, endothelial cells (EC's), and monocytes[7]. It is a known chemo attractant for monocytes, macrophages, eosinophils, and lymphocytes, and is also a ligand for CC chemokine receptor 2 (CCR2)[7,8]. MCP-1 is thought to mediate angiogenesis *via* recruitment of proangiogenic protein producing monocytes and macrophages and endothelial cells into wounds and tumors. The chemotaxis of EC'ss is inhibited by MCP-1 antibodies *in vitro* and *in vivo*[9]. MCP-1, by binding to CCR2 on the surface of EC's, has also been shown to promote EC migration, which is a critical early step in angiogenesis[9,10]. There also appears to be an intimate relationship between MCP-1 and VEGF. Interestingly, VEGF increases MCP-1 mRNA expression in EC *in vitro* cultures[11,12]. Also, there is evidence that MCP-1 modulates VEGF's effects; MCP-1 antibody diminishes VEGF mediated tubule formation in angiogenesis assays[12].

Angiogenesis is fundamental to both wound healing and tumor growth. MCP-1 is found abundantly during the initial inflammatory stage of wound healing[9], where it plays a role in recruiting monocytes and macrophages[11,12]. Weber *et al*[10] showed that the presence of a MCP-1 receptor antagonist or neutralizing MCP-1 antibody impaired the ability of ECs to migrate and close wounds, whereas the addition of MCP-1 facilitated repair. Thus, MCP-1 appears to induce EC migration during wound repair[10]. Additionally, endothelial MCP-1 secretion is increased in the setting of multiple wounds[10]. Finally, wound re-epithelialization is significantly delayed in MCP-1 knockout mice[13].

There is also experiment evidence suggesting that MCP-1 plays a role in tumor growth. Nakashima *et al*[14] demonstrated that transfection of MCP-1 into a murine CRC cell line promoted lung metastases by augmenting neovascularization. Further, Salcedo *et al*[9] showed that treatment of immunodeficient mice, in whom metastases had been established *via* inoculation with human breast carcinoma cells, with administration of a neutralizing antibody to MCP-1 resulted in significant longer survival and decreased growth of lung micrometastases[9]. MCP-1 has also been associated with multiple human cancers. A study of breast cancer patients revealed high levels of MCP-1 expression in primary breast cancers by enzyme linked immuno-absorbent assay (ELISA) and immunohistochemical analysis; this expression correlated significantly with macrophage accumulation in the tumors[15]. In another study, patients with primary and recurrent ovarian cancer were shown to have significantly higher MCP-1 serum levels compared to patients with benign ovarian pathology[16]. Furthermore, MCP-1 serum levels have been shown to correlate with histological grade in ovarian cancer patients[16].

The impact of CRC on plasma levels of MCP-1 is unknown. Further, the effect of minimally invasive colorectal resection (MICR) on postoperative (PostOp) plasma MCP-1 levels is unknown. MCP-1 may contribute to the overall proangiogenic state of plasma noted following surgery. The purpose of this study was twofold: (1) to assess plasma levels of MCP-1 before surgery in CRC and BEN disease patients; and (2) to determine levels after MICR for cancer.

MATERIALS AND METHODS

Study population

Consenting patients with CRC or benign colorectal

disease (BEN) that underwent elective MICR during the period of 2003-2011 were identified from a larger population of patients who had been enrolled in an IRB-approved multicenter prospective data and blood banking protocol. The broadly stated purpose of this effort is to study the physiologic, immunologic, and oncologic ramifications of major abdominal surgery. Enrolled patients underwent surgery alone and did not receive a novel drug or other therapy. The indications and type of surgery as well as the demographic, operative, and short term recovery data was prospectively collected for all patients. Recently transfused patients, immunosuppressed patients (medication-related, HIV+, *etc.*), and those who received radio- or chemotherapy within 6 wk of surgery were excluded. Patients undergoing urgent or emergent surgery were, likewise, excluded.

Blood sampling and processing

To be eligible for entry into this study plasma samples for the following time points needed to be available for CRC patients who underwent MICR: preoperative (PreOp), postoperative day (POD) 1, POD 3, and at least 1 later postoperative specimen from POD 7-28. Of note, blood samples after POD 7 were obtained at follow up office appointments but were not scheduled on a specific POD. Many patients refused late blood draws. Because the number of specimens on any given late postoperative day was small it was necessary to "bundle" the specimens from 7 d time blocks (POD 7-13, 14-20, 21-27) and consider these as single time points. PreOp blood samples were obtained prior to surgery and processed in an identical manner for comparison of MCP-1 levels in CRC patients and the BEN group. Samples were collected in heparin-containing tubes, were processed within 5-6 h of collection. After centrifugation, the plasma was frozen and stored at -80 ˚C until the assays were performed.

Plasma MCP-1 determination

Plasma levels of MCP-1 were determined in duplicate using a commercially available enzyme linked immunoabsorbent assay (R and D Systems, Minneapolis, MN) according to the manufacturer's instructions. MCP-1 concentrations (pg/mL) were calculated using a standard curve made in every assay and were reported as median and 95% confidence intervals for the PreOp *vs* PostOp MCP-1 comparisons, the preoperative CRC *vs* BEN group comparison, and for the Stage 1-3 CRC sub group comparisons.

Statistical analysis

Demographic and clinical data are expressed as the mean and SD for continuous variables. Preoperative MCP-1 values in the cancer and Benign populations were not normally distributed and, thus, the median values for each group were calculated and compared using the Mann and Whitney *U* test. In regards to the CRC Pre *vs* Postoperative MCP-1 comparisons, the results are reported as the median and 95%CIs and the Wilcoxon paired test was used to analyze the data. Significance was set at $P < 0.01$ (Bonferroni adjustment was applied). In regards to the sub group comparisons of preoperative MCP-1 values *vs* the stage 1-3 CRC subgroups, the results are reported as the median and 95%CIs and the Mann and Whitney *U* test was utilized for the analysis. Correlation between postoperative MCP-1 plasma levels *vs* incision size and length of surgery was evaluated by the Spearman's rank correlation coefficient (rs) and the correlation between complication rate and PostOp MCP-1 levels was calculated *via* logistic regression analysis. All data analysis was performed using SPSS version 15.0 (SPSS, Inc., Chicago, IL).

RESULTS

Overall, a total of 102 CRC patients (59 males, 43 females with a mean age of 67.1 ± 12.3 years) were included in the study. Seventy patients (69%) had colon cancers while 32 (31%) had rectal lesions. The final cancer stage breakdown is as follows: Stage Ⅰ, 30.5%; Stage Ⅱ, 30.5%; Stage Ⅲ, 37%; and Stage Ⅳ, 2%. The majority of patients (66%) underwent laparoscopic-assisted (LA) resections, whereas 34% had a hand-assisted or hybrid laparoscopic (HAL) procedure. The types of resection performed, as well as other operative data are provided in Table 1. The overall complication rate for the CRC patients was 21% and there were no anastomotic leaks, intra-abdominal abscesses, or perioperative deaths. The complications noted included the following: wound infections (2 patients); cardiac (2); pulmonary (3); ileus (6); urinary retention (5); SBO (3); and *C. difficile colitis* (1).

A group of 86 benign colorectal disease patients (BEN) who underwent MICR served as the control group for the preoperative MCP-1 levels comparison. The indications for MICR in the BEN group were diverticulitis ($n = 30$) and benign neoplasms ($n = 56$). The CRC group was significantly older than the BEN group (67.1 ± 12.3 *vs* 59.3 ± 13.4 years, $P < 0.0001$; Table 1) but with similar male to female ratios.

Preoperative MCP-1 plasma levels in CRC vs BEN group

The median PreOp MCP-1 plasma level in the CRC patients (283.1, CI: 256.0, 294.4) was modestly but significantly higher (24%) than the level noted in the BEN patient group (227.5, CI: 200.2, 245.2; $P = 0.0004$; Figure 1).

Preoperative MCP-1 plasma levels in the Stage 1-3 CRC subgroups

In regards to final cancer stage, the median PreOp values for the Stage 1 to 3 CRC groups were as follows: Stage Ⅰ, 296.5 (CI: 231.2, 343.7); Stage Ⅱ, 274.2 (CI: 217.3, 292.2); and Stage Ⅲ, 285.9 (CI: 251.1, 296.9). Although the results for each Stage group (1-3) were significantly higher than the BEN group's median value, there was no significant difference amongst the Stage 1, 2, and 3 groups [Note: There were too few Stage 4 patients ($n = 2$) in the CRC population to permit statistical analysis].

Table 1 Demographic and clinical characteristics of the study population

	Cancer (n = 102)
Age, yr (mean ± SD)	67.1 ± 12.3
Sex (n)	
Male	59
Female	43
Incision length, cm (mean ± SD)	7.1 ± 2.8
Operative time, min (mean ± SD)	266.5 ± 113
Length of stay, d (mean ± SD)	5.9 ± 2.3
Type of resection	
Right	39 (38%)
Transverse	4 (4%)
Left	8 (8%)
Sigmoid/Rectosigmoid	14/4 (18%)
LAR/AR	24/2 (25%)
APR	3 (3%)
Subtotal/total	2/2 (4%)
Surgical method	
Laparoscopic-assisted	67 (66%)
Hand-assisted/hybrid laparoscopic	35 (34%)

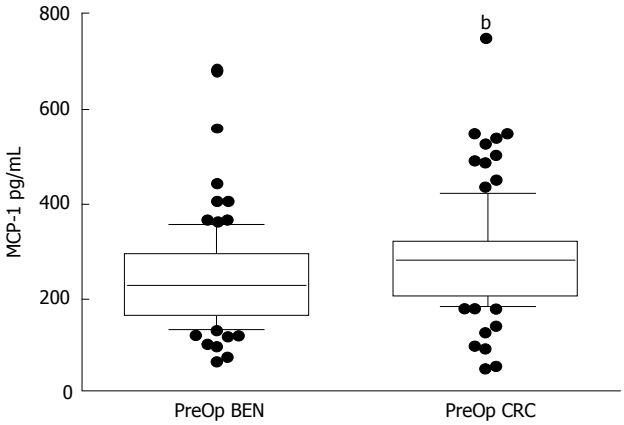

Figure 1 Enzyme linked immuno-absorbent assay determined preoperative plasma monocyte chemotactic protein-1 levels of patients in the benign and malignant group. Monocyte chemotactic protein-1 (MCP-1) levels are expressed as median and CI. [PreOp Benign (n = 86) *vs* PreOp Cancer (n = 102), ^{b}P = 0.0004]. PreOp: Preoperative; BEN: Benign disease; CRC: Colorectal cancer.

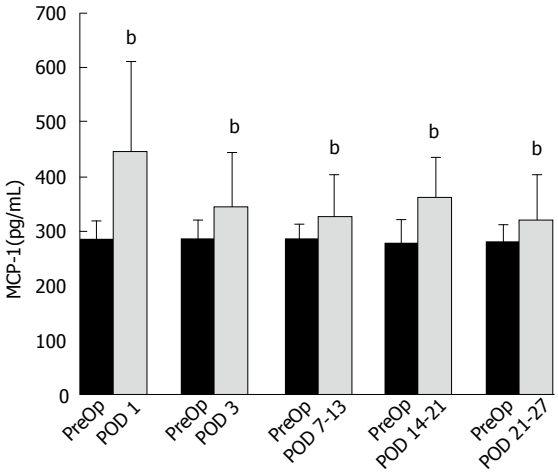

Figure 2 Enzyme linked immuno-absorbent assay determined preoperative and postoperative monocyte chemotactic protein-1 levels of colorectal cancer patients. Monocyte chemotactic protein-1 (MCP-1) levels are expressed as expressed as median and 75% quartile range [PreOp *vs* POD 1 (n = 102), PreOp *vs* POD 3 (n = 100), PreOp *vs* POD 7-13 (n = 61), PreOp *vs* POD 14 -20 (n = 27), PreOp *vs* POD 21-27 (n = 28), ^{b}P < 0.001]. PreOp: Preoperative; POD: Postoperative day.

Comparison of Pre vs postop MCP-1 plasma levels in CRC patients

The median PreOp MCP-1 level in CRC patients was 283.1 (CI: 256.1, 294.4) pg/mL (n = 102). When compared to PreOp levels, significantly elevated mean MCP-1 plasma levels (pg/mL) were observed on POD 1 (446.3, CI: 418.0, 520.1; n = 102, P < 0.001), POD 3 (342.7, CI: 320.4, 377.4; n = 100, P < 0.001), POD 7-13 (326.5, CI: 299.4, 354.1; P < 0.001), POD 14-20 (361.6, CI: 287.8, 407.9; n = 27; P < 0.001), and POD 21-27 (318.1, CI: 287.2, 371.6; n = 28; $P \leq$ 0.001). Because the "n" for the POD 3 and later time points was less than 102 and unique for each time point, the PreOp baseline level for each of these time points was somewhat different. This is reflected in Figure 2, which provides in bar graph form the mean PreOp baseline for each postoperative time point.

The percent increase over the PreOp baseline for each postoperative time point is as follows: POD 1 (73.5%); POD 3 (37.2%); POD 7-13 (24.6%); POD 14-20 (39.7%); and POD 21-27 (25%).The percentage of CRC patients that had plasma levels increased from the median PreOp baseline levels of each subgroup were: POD 1 (79%); POD 3 (81%); POD 7-13 (73.8%); POD 14-20 (89%); and POD 21-27 (89.3%).

Correlation of post-operative plasma MCP-1 levels vs incision length and length of surgery

There was a weak correlation between plasma MCP-1 levels on POD1 and the incision length (r_s =0.217, P = 0.006) as well as the length of surgery (r_s = 0.268, P = 0.007). There was no such correlation noted for the 4 other postoperative time points in regards to incision or operation length. Also, there was no correlation found between the presence of complication(s) and the degree of the postoperative plasma MCP-1 elevation at any of the postoperative time points.

DISCUSSION

The median preoperative plasma level of MCP-1 was significantly higher in the CRC patients than in the BEN disease group. This suggests that, in some patients, the tumor is generating MCP-1, either directly or indirectly. Unfortunately, this study did not include tumor analysis (microarray or RT-PCR) and thus, we can only speculate as to the origin of the additional MCP-1. Of note, no correlation was identified between PreOp levels and tumor stage.

In regards to the comparison of PreOp and Postop MCP-1 levels in the CRC patients, plasma levels were

significantly elevated for the first month after MICR. The greatest increase was observed during the first week after surgery. MCP-1, therefore, joins the list of proteins whose blood levels are altered after MICR. The vast majority of surgery-related blood protein alterations (CRP, IL-6, IL-2, FGF, HGF, angiostatin, endostatin, *etc*.) are short lived and resolve within the first 3 to 5 d after surgery. Of note, the percent change from base line in regards to MCP-1 is amongst the highest when compared to the previously mentioned proteins. Many of the short duration blood compositional changes are related to the acute phase inflammatory response to surgical trauma as well as to the anesthesia. Because blood levels were increased during the entire first month after surgery, MCP-1 joins the small list of proteins (VEGF, Ang-2, PlGF, and sVCAM) with long duration plasma elevations; interestingly, all of these proteins play a role in angiogenesis[2-5]. MCP-1 facilitates angiogenesis through several mechanisms, including its intimate relationship with VEGF[17,18].

Interestingly, VEGF increases MCP-1 mRNA expression in endothelial cell *in vitro* cultures[17,18]. Also, there is evidence that MCP-1 modulates VEGF's effects; MCP-1 antibody diminishes VEGF mediated tubule formation in angiogenesis assays[18]. Collectively; the above mentioned group of proteins play a role in the early stages of neovascularization and most modulate VEGF's effects. What is the source of the plasma MCP-1 increases after MICR?

The authors believe that the tumor produced MCP-1 is not responsible for the postoperative increases in plasma MCP-1 levels. Logically, the blood levels should decrease after resection if the source of the added MCP-1 was the tumor. The significant correlation observed between POD1 MCP-1 levels and incision length and length of surgery suggests that the MCP-1 levels on POD 1 could be attributed, in part, to the surgical stress and the initial inflammatory response which takes place early after surgery. Of note, no such correlation was found from POD 3 onward. It is the authors' opinion that the sustained plasma MCP-1 elevation is related to wound healing. MCP-1, in wounds, accelerates macrophage trafficking into inflammatory foci and also plays a role in angiogenesis. Angiogenesis is critical to wound healing which is a lengthy process that lasts, at least, 6 to 8 wk. There is evidence that VEGF levels in wounds are very high; it is assumed that some of the wound VEGF finds its way into the blood, raising plasma concentrations[19-21]. Although unproven, the authors believe it is likely that wound levels of the other proangiogenic proteins, including MCP-1, whose blood levels are persistently increased after surgery are also notably increased.

Interestingly, as mentioned earlier, it has been demonstrated *via* EC cultures that plasma from the second and third weeks after MICR stimulates EC proliferation (specifically, branch point formation which is the culture equivalent of microtubule formation), migration, and invasion when compared to culture results obtained with preoperative plasma. These EC functions are critical early steps in the process of neovascularization, critical to both wound healing and solid tumor growth beyond 2 mm[22]. Similar EC culture results were noted when plasma from open CRC resection patients was similarly assessed. What are the possible ramifications, if any, of the proangiogenic postoperative plasma?

In the proportion of patients that harbor residual micrometastases the proangiogenic postoperative plasma changes may promote tumor growth. Persistently elevated levels of MCP-1 after MICR for CRC may promote recurrence in patients who harbor tumor micro foci. The complex process of residual tumor growth and metastasis may be supported by other angiogenic proteins whose blood levels remained elevated after MICR for CRC such as VEGF, PLGF, sVCAM-1 ANG2 and MMP3. There are case reports of rapid tumor growth and the development of metastases in cancer patients who undergo major surgery[23,24]. Of note, there is also experimental evidence that laparotomy and bowel resection, in the murine setting, in general, are associated with increased rates of systemic tumor establishment and growth postoperatively[25-27]. Furthermore, human postoperative serum from POD 1 has been shown to stimulate *in vitro* growth of human colon cancer cells when compared to culture results obtained with preoperative plasma[28]. It is also well documented that surgery induces transient postoperative cell-mediated immune suppression. In addition, surgery also impairs lymphocyte and neutrophil chemotaxis, macrophage function, and delayed type hypersensitivity responses[29-31]. These changes might impact early postoperative tumor growth as well.

Thus, the first month after surgery may be a dangerous time for cancer patients. Standard adjuvant chemotherapy is most often started 4 to 8 wk after surgery because of fears that earlier administration may inhibit wound and anastomotic healing. Perhaps, the logical next step is to search for anti-cancer drugs that could be safely given during the first month following surgery to serve as a bridge between "curative" resection and the start of adjuvant chemotherapy. The ideal agent would effectively target tumor cells that remain after surgery without interfering with wound or anastomotic healing. The authors have done one human and numerous murine studies that have assessed the anti-cancer impact of perioperative administration of a number of immunomodulatory and anti-cancer agents[32-34].

One weakness of the present study is the limited number of blood samples obtained beyond the first postoperative week. The majority of these samples were obtained during office follow up visits, which were scheduled at the discretion of the patient. Additionally, many patients refused to have late samples drawn. Therefore, it was impossible to obtain blood samples on a set postoperative timeline. To permit statistical analysis, late samples were bundled into 7-d blocks and considered as single time points. Given the limited number of postoperative samples obtained after the first postoperative month, we were also not able to determine when MCP-1 levels re-

turn to baseline.

At baseline, plasma MCP-1 levels are significantly elevated in CRC patients. Also, for at least 1 mo after minimally invasive tumor resection, plasma MCP-1 levels are significantly elevated from the preoperative baseline. The early postoperative elevations (1st week) may be related to the acute inflammatory response associated with surgical trauma and anesthesia. Although unproven, it is believed that the elevations observed during weeks 2 through 4 are related to wound healing. MCP-1 joins the growing list of pro-angiogenic proteins whose blood levels are persistently elevated after colorectal resection (VEGF, PlGF, sVCAM, ANG-2, MMP-3, *etc.*). These surgery-related plasma compositional changes may stimulate the growth of residual micrometastases early after resection. Further investigations are needed to determine the clinical ramifications, if any, of these transient yet significant changes. The search for and administration of anti-cancer agents that do not inhibit wound healing may be indicated.

COMMENTS

Backgrounds

Blood levels of proangiogenic proteins are increased after minimally invasive colorectal cancer resection. Postoperative plasma enriched in proangiogenic proteins promotes angiogenesis *in vitro*. The angiogenic proteins in question [vascular endothelial growth factor (VEGF), angiopoetin-2 (Ang-2), placental growth factor (PlGF), soluble vascular adhesion molecule-1 (sVCAM-1) and Matrix metalloproteinase 3 (MMP-2)] have been noted to be significantly elevated for 2-4 wk following minimally invasive colorectal resection for CRC. Monocyte chemotactic protein-1 (MCP-1) has documented proangiogenic effects, however, little is known about plasma MCP-1 levels preoperatively in CRC and benign disease patients or in CRC patients after MICR.

Research frontiers

MCP-1, a member of the C-C chemokine family, is expressed by some cancers and has been shown to support tumor angiogenesis and development. MCP-1 is thought to mediate angiogenesis *via* recruitment of proangiogenic protein producing monocytes and macrophages and endothelial cells into wounds and tumors. The authors evaluated preoperative and post-MICR MCP-1 levels in CRC patients. The concern is that significantly elevated blood levels of MCP-1 perioperatively may enhance the plasma's proangiogenic properties during the first month after surgery which, in turn, may promote tumor angiogenesis in residual lesions.

Innovations and breakthroughs

Previous studies have established that significant elevations in plasma levels of VEGF, Ang-2, PlGF, sVCAM-1 and MMp-3 occur for 2-4 wk following MICR for CRC. Additionally, prior studies have shown that postoperative plasma from cancer patients stimulates *in vitro* endothelial cell (EC) proliferation, migration, and invasion, all of which are critical steps in angiogenesis and tumor development. This study found elevated levels of plasma MCP-1, a protein with proangiogenic effects, before and for 1 mo after surgery. Collectively, the sustained elevations in blood levels of the above mentioned group of proangiogenic proteins may support metastasis formation and the growth of residual tumors.

Applications

This study further supports the concept that surgery-related stress and post-surgery wound healing related plasma compositional changes may stimulate the growth of residual micrometastases early after resection. The search for and administration of anti-cancer agents during the perioperative period appears warranted; agents used in this time from must not inhibit wound healing.

Terminology

It has earlier been shown that both MICR and open colorectal resection are associated with sustained (2-4 wk after surgery) plasma protein changes that collectively enhance the angiogenic properties of plasma. These changes, thought to be related to wound healing, may support tumor angiogenesis early after surgery. This study shows that plasma levels of MCP-1, another proangiogenic protein, are elevated after MICR for a month. Thus, another proangiogenic protein is added to the list. Collectively, these prolonged blood elevations may support the growth of residual cancer and initiation of cancer by circulating tumor cells.

Peer review

This study is interesting and I would like to give my suggestions to impact the authors understanding of the tumor tissue in the elucidation of aberrant molecular aspect changes in the tumor microenvironment and surgical margins to impact the paper.

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REVIEW

Usefulness of endoscopic ultrasound-guided fine needle aspiration in the diagnosis of hepatic, gallbladder and biliary tract Lesions

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Abstract

Endoscopic ultrasound (EUS)-guided fine needle aspiration (FNA) of the liver is a safe procedure in the diagnosis and staging of hepatobiliary malignancies with a minimal major complication rate. EUS-FNA is useful for liver lesions poorly accessible to other imaging modalities of the liver. EUS-guided FNA of biliary neoplasia and malignant biliary stricture is superior to the conventional endoscopic brushing and biopsy.

Key words: Endoscopic ultrasound; Fine needle aspiration; Hepatocellular carcinoma; Bile duct stricture; Gallbladder; Cholangiocarcinoma; Biliary drainage

Core tip: The present article reviews the usefulness of endoscopic ultrasound-guided fine needle aspiration (EUS-FNA) in patients with focal liver and biliary tract lesions. We conducted MEDLINE search using the terms "endoscopic ultrasound-guided fine needle aspiration", "focal liver lesions" and "biliary tract lesions", "EUS and biliary stricture", EUS and focal liver mass", "EUS and cholangiocarcinoma" and "EUS and gallbladder" to retrieve articles published between 1999 to 2014.

Hammoud GM, Almashhrawi A, Ibdah JA. Usefulness of endoscopic ultrasound-guided fine needle aspiration in the diagnosis of hepatic, gallbladder and biliary tract Lesions. *World J Gastrointest Oncol* 2014; 6(11): 420-429 Available from: URL: http://www.wjgnet.com/1948-5204/full/v6/i11/420.htm DOI: http://dx.doi.org/10.4251/wjgo.v6.i11.420

INTRODUCTION

Endoscopic ultrasonography (EUS) has become an indispensable diagnostic and therapeutic procedure in the field of gastroenterology coupling endoscopy with high frequency echo sonography. Endoscopic ultrasound-guided fine needle aspiration (EUS-FNA) is performed using the curved linear array echoendoscope (Figure 1) using various needles (Figure 2). The recently introduced forward viewing linear echoendoscope is gaining momentum in endoscopic ultrasound-guided interventions (Figure 1). EUS-FNA is minimally invasive that is utilized for procurement of tissue from unresectable tumors. EUS-guided fine needle aspiration is used increasingly for the diagnosis of mediastinal, pancreatic and gastric tumors, however, not much is known about EUS-FNA in hepatic lesions. EUS imaging of the liver is currently limited to the left lobe, the proximal right lobe, the hilum and part of the intrahepatic biliary tract. EUS-FNA may be considered as an alternative to liver percutaneous biopsy in patients at high risk of bleeding or with small lesions of the liver uncharacterized by cross-sectional abdominal imaging. EUS-guided biliary drainage (EUS-BD) was developed using a curved linear array echoendoscope for cases with failed endoscopic biliary drainage. Table 1 summarizes the use of endoscopic ultrasound-guided

Table 1 Summary of the use of endoscopic ultrasound-guided fine needle aspiration in the diagnosis of hepatic and biliary tract lesions

Diagnosis of focal malignant and benign liver lesions
Diagnosis of malignant biliary stricture and neoplasia
Preoperative staging of hepatocellular carcinoma and lymph node metastasis
Ablation of focal malignant and benign liver lesions
Liver biopsy
Fluid acquisition and biopsy of peritoneal and omental deposits
Drainage of intrahepatic and extrahepatic biliary tree
Drainage of hepatic abscesses

fine needle aspiration in the diagnosis and management of hepatic, gallbladder and biliary tract lesions.

FEASIBILITY OF ENDOSCOPIC ULTRASOUND-GUIDED FINE NEEDLE ASPIRATION OF FOCAL LIVER LESIONS

Focal liver lesions include simple liver cyst, focal nodular hyperplasia, hepatic adenoma, hepatic hemangioma, regenerative nodular hyperplasia, biliary cystadenoma, intrahepatic cholangiocarcinoma, hepatocellular carcinoma and metastatic liver lesions. The majority of these lesions can be diagnosed with certainty by cross-sectional abdominal imaging and by percutaneous liver biopsy. However, small lesions less than 1-cm in diameter may not be well characterized by abdominal ultrasound (US), computed tomography (CT) and/or magnetic resonance imaging (MRI). In general, the lowest ultrasound frequency available should be used to maximize penetration. EUS-guided liver biopsy using a 19-gauge FNA needle (non-Trucut) and EUS-guided Trucut needle appear to be feasible, safe and provide excellent diagnostic yield and specimen adequacy[1-3]. In a retrospective study by DeWitt *et al*[4], EUS-FNA of liver lesions that range from 3-40 mm in size was performed in 77 patients[4]. Of these lesions, 58% were diagnostic for malignancy, 33% were benign, and 9% were nondiagnostic. In a study by tenBerge *et al*[5], EUS-FNA was used to sample liver lesions in 167 patients. The indications were pancreatic mass in 37%, liver metastasis of unknown origin in 20%, esophageal, gastric and liver masses. EUS-FNA of the liver revealed malignancy in patients when abdominal ultrasonography-guided FNA and CT-guided FNA have failed. Crowe *et al*[6] compared 34 percutaneous computerized tomographic-guided fine needle aspiration liver biopsies and 16 EUS-FNA liver biopsies showed comparable results. These studies and others suggest that EUS-FNA is feasible and comparable to US/CT-guided biopsy in the diagnosis of patients with focal liver lesions.

Malignant focal/metastatic liver lesions

EUS can provide high resolution imaging of the left hepatic lobe to detect unsuspected metastatic disease during staging and may deter from unnecessary surgery[7,8]. EUS-FNA of liver lesions can provide useful information for future management. Hepatic metastasis is generally echo-poor without a distinct border such as the one seen in pancreatic and colon metastasis (Figure 3) or echo-rich such as seen in metastatic neuroendocrine tumors and renal cell carcinoma (Figure 4). EUS-FNA can detect tumors less than 3 mm in size[7]. Solid liver lesions accessible by EUS may be safely sampled by EUS-FNA. The use of stylet during FNA does not appear to confer any advantage with regards to the adequacy of specimen or diagnostic yield of malignancy[9]. In a prospective study of 132 subjects with newly diagnosed tumors, the diagnostic accuracy of EUS/EUS-FNA and CT scan in detecting hepatic metastasis was 98% and 92%, respectively ($P = 0.0578$)[10]. In a large single-center experience, the sensitivity of EUS-FNA for the diagnosis of liver cancer ranged from 82% to 94%[4]. In a prospective study of 41 patients, 33 of whom had clinical findings suggestive of liver malignancies, EUS-FNA provided biopsy specimens in 40/41 patients[11]. Combining histological and cytological features had a sensitivity of 94%, specificity of 100%, negative predictive value of 78%, and positive predictive value of 100%[11]. These data suggest that EUS-FNA is a sensitive diagnostic procedure in patients with focal malignant liver lesions especially to those confined to left hepatic lobe.

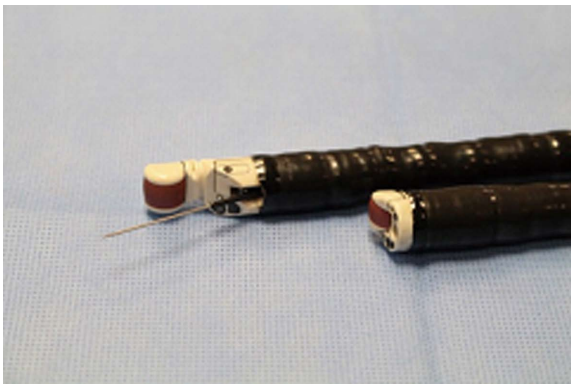

Figure 1 The curved linear array videoechoendoscope (GF-UCT180) (Back); The new prototype forward viewing linear array videoechoendoscope (TGF-UC180J) (Front).

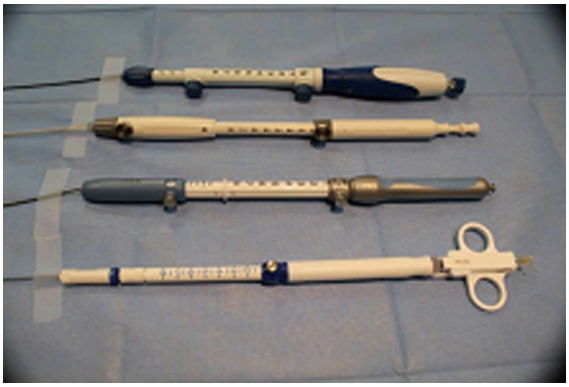

Figure 2 Various echoendoscopic needles used for fine needle aspiration.

Hepatocellular carcinoma

EUS-FNA may be useful in the diagnosis of focal liver lesions, early hepatocellular carcinoma, and evaluation of

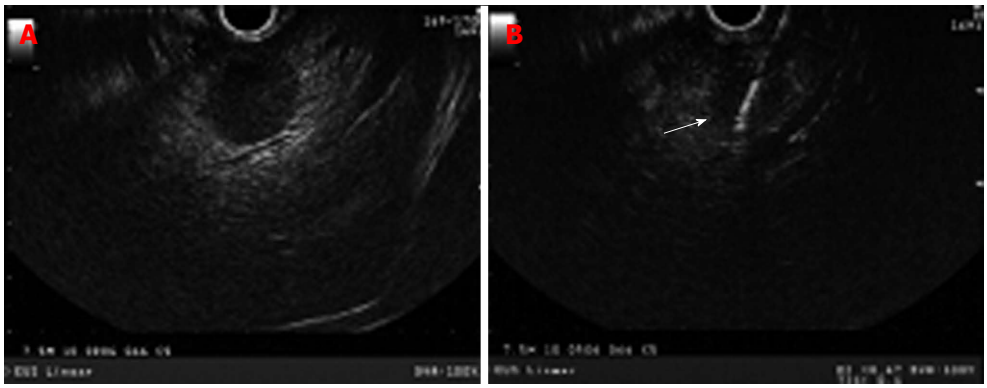

Figure 3 Curved linear echoendoscope showing a rounded hypoechoic left lobe liver lesion with no well-defined border representing liver metastasis in a patient with pancreatic adenocarcinoma (A); fine needle aspiration (white arrow) was performed using 22 gauge needle (B).

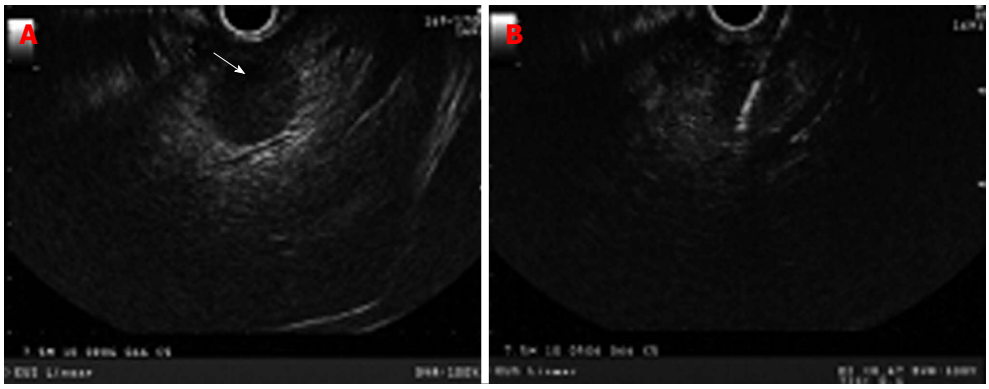

Figure 4 Hyperechoic rounded liver lesion (white arrow) representing a metastasis in patient with pancreatic neuroendocrine tumor with biliary obstruction and dilated intrahepatic duct (A); endoscopic ultrasound-guided fine needle aspiration of liver lesion using 22 gauge needle (B).

perihepatic adenopathy[12-15]. Hepatocellular carcinoma (HCC) may appear on EUS images either as hypoechoic or hyperechoic[16]. Burrel *et al*[17] showed that lesions smaller than 1cm in diameter are missed in a significant percentage (70%) of the patients by modalities such as CT imaging[14,18] and magnetic resonance imaging[18]. EUS and EUS-FNA are particularly valuable for the preoperative staging of hepatocellular and metastatic liver carcinoma. In a study by Awad *et al*[18], EUS identified liver lesions 0.3-14 cm in size in all 14 study patients with hepatocellular cancer and metastatic lesions who underwent both dynamic CT scans and EUS[18]. Moreover, in 28% of the patients, EUS identified new lesions less than 0.5 cm in size. In a prospective single-center study evaluating 17 patients who underwent cross-sectional imaging and EUS, 9 had liver tumors[16]. EUS-FNA established a tissue diagnosis in 8 of the 9 cases. The diagnostic accuracy of transabdominal ultrasonography, abdominal CT, MRI, and EUS/EUS-FNA were 38%, 69%, 92%, and 94%, respectively[16]. Another retrospective study evaluated the sensitivity and complications of EUS-FNA of liver nodules in 14 patients, performed by single endoscopist[19]. Twenty-one percent of the cases were hepatocellular carcinoma. The sensitivity of diagnosis of malignant liver lesions utilizing cytology was 78.5%. However, combining clinical course and pathology increased the sensitivity to 100%. These data suggest that EUS has an excellent diagnostic accuracy in patients with HCC.

Moreover, EUS-guided fine needle aspiration of portal vein thrombus to detect malignancy has been described in literature[20,21]. More recently, a newly developed promising technique utilizing real time-sonoelastography (RTE) by EUS might improve the characterization and differentiation between benign and malignant focal liver lesions[22].

Screening and treatment of HCC

The use of EUS-FNA in screening for HCC is limited by the semi-invasive nature of the procedure as well as its inability to evaluate all liver segments at this time[13]. Nevertheless, EUS can provide an additional option for treatment in patients with hepatocellular carcinoma who are difficult to treat utilizing percutaneous ablative therapy such as endoscopic ultrasound-guided ethanol injection[23,24] and EUS-guided Nd:YAG laser ablation of a caudate lobe hepatocellular carcinoma[25].

Benign focal liver lesions

Large hepatic cysts are amenable to percutaneous drainage or surgical resection. EUS-guided ethanol injection has been shown to be effective in treating patients with large hepatic cysts especially in the left hepatic lobe. In a retrospective study evaluating 17 patients with 19 hepatic cysts (median cyst volume before therapy was 368.9 mL)[26], ten cysts were drained by the percutaneous

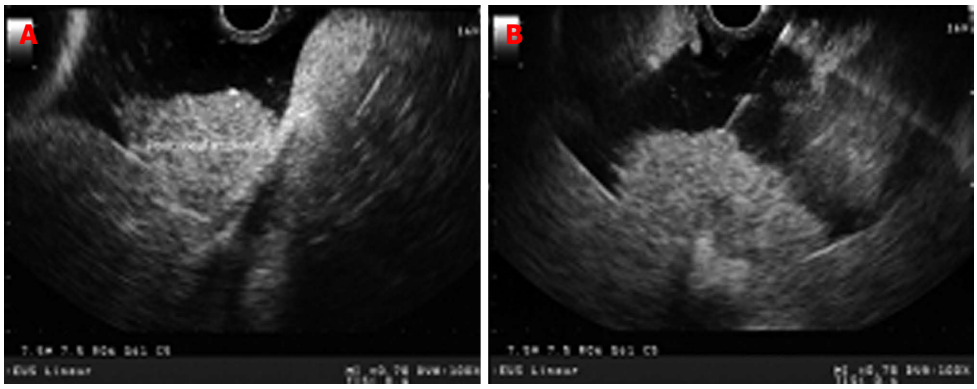

Figure 5 Peritoneal deposits in a patient with malignant ascites. Peritoneal implants appear as hypoechoic in comparison to the surrounding tissue but hyperechoic in comparison to the anechoic ascitic fluid (A); endoscopic ultrasound-guided fine needle aspiration of a large peritoneal deposit (B).

approach and 8 cysts underwent EUS-guided aspiration and lavage treatment. During 15-mo follow-up, the cysts showed nearly 100% reduction in the EUS-guided group compared to 97% reduction in the percutaneous group. Furthermore, EUS-FNA has also shown excellent success rates in selected patients with hepatic abscesses. In a recent review of the literature by Singhal *et al*[27], seven studies have reported 100% technical and clinical success rates of EUS-guided drainage of hepatic abscesses in patients refractory or not amenable to percutaneous drainage.

Ascites and peritoneal metastasis

EUS-guided paracentesis is valuable in the cytologic diagnosis and staging of malignant ascites[28,29]. EUS frequently identifies ascites missed by other imaging modalities and may identify malignancy[30]. It is particularly useful when CT imaging does not identify abnormalities[31]. EUS-FNA can be performed safely for therapeutic paracentesis[32]. In a retrospective single center study that evaluated 101 patients who underwent EUS-guided paracentesis, the specificity, sensitivity, positive and negative predictive values, and diagnostic accuracy were 100%, 80%, 100%, 95% and 96%, respectively[29]. Furthermore, EUS-FNA can be used effectively and safely to obtain tissue from the peritoneum for diagnosis of tuberculous peritonitis[33]. EUS-FNA allows the sampling of peritoneal metastatic lesions, which appear on EUS images as hyperechoic compared to surrounding anechoic ascitic fluid (Figure 5). In a small study involving 12 patients with undiagnosed ascites, peritoneal deposits noted in 10 (83.3%) patients[34]. The cytological results were positive for malignancy in 6 of those patients, while the remaining four patients had inflammatory cells.

ENDOSCOPIC ULTRASOUND-GUIDED FINE NEEDLE ASPIRATION OF BILE DUCT, GALLBLADDER AND AMPULLARY LESIONS

Cholangiocarcinoma and proximal biliary strictures

Preoperative tissue diagnosis is required for hilar neoplasia [cholangiocarcinoma (CCA)] to avoid risk of unnecessary extensive surgery. Endoscopic transpapillary brush cytology and forceps biopsy are used for the pathological diagnosis of malignant biliary strictures. Endoscopic retrograde cholangiography (ERC) is currently the main diagnostic procedure performed to obtain sampling of the biliary tree. However, the sensitivity and specificity of obtaining a sample in biliary neoplasia is variable. EUS is capable of visualizing the hilum at the duodenal bulb by tracing the common bile duct (CBD) towards the liver hilum. In a meta-analysis of 36 studies by Garrow *et al*[35] EUS has a sensitivity of 78% and specificity of 84% in detecting malignant biliary strictures. Nayar *et al*[36] reported on 32 patients who underwent 36 procedures for hilar lesions. The overall sensitivity, accuracy, specificity, positive predictive value and negative predictive value of EUS-FNA were 52%, 68%, 100%, 100% and 54%, respectively. Fritscher-Ravens *et al*[37] prospectively evaluated 44 patients with hilar strictures diagnosed by CT and/or Endoscopic retrograde cholangiopancreaticography (ERCP) that were suspicious for hilar cholangiocarcinoma but had inconclusive tissue diagnosis. The sensitivity, accuracy, and specificity of EUS-FNA in this study were 89%, 91%, and 100%, respectively. Moreover, EUS and EUS-FNA changed preplanned surgical approach in about half of these patients[37]. The above studies suggest that hilar neoplasia can be sampled by EUS-FNA although the accuracy and sensitivity were not robust. Moreover, EUS-FNA may be considered in evaluating regional lymph nodes to evaluate for metastasis in patients with unresectable hilar cholangiocarcinoma[38,39]. EUS-FNA in patients with cholangiocarcinoma did not appear to adversely affect the overall survival[40].

Distal malignant biliary stricture

The sensitivity of EUS-FNA is much higher in distal malignant biliary strictures than proximal strictures. Malignant distal biliary strictures are most commonly secondary to pancreatic malignancy and/or distal bile duct cholangiocarcinoma (Figure 6). In a recent prospective comparative one-year study of 51 patients who underwent EUS and ERCP in the same session for evaluation of malignant biliary obstruction[41], EUS-FNA was supe-

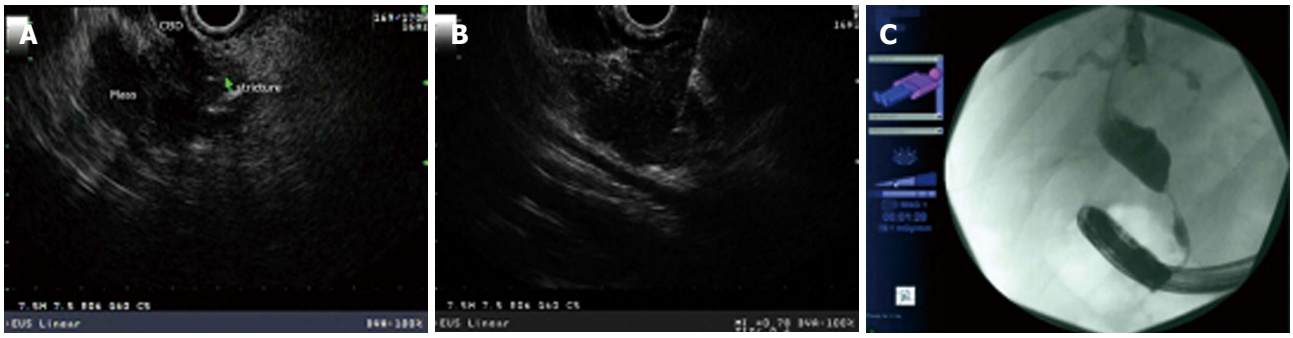

Figure 6 Malignant distal biliary strictures are most commonly secondary to pancreatic malignancy and/or distal bile duct cholangiocarcinoma. A: Distal common bile duct stricture secondary to a large heterogenous hypoechoic pancreas head mass with irregular border; B: Endoscopic ultrasound-guided fine needle aspiration of pancreas head mass/stricture; C: Distal irregular common bile duct stricture seen on cholangiogram.

rior to ERCP in tissue sampling for evaluating suspected malignant biliary obstruction, especially for pancreatic masses with an overall accuracy and sensitivity of 94% and 94% for EUS-FNA, and 53% and 50% for ERCP sampling, respectively. In an observation study of prospectively collected data of 228 patients with biliary strictures who underwent EUS[42]. Cholangiocarcinoma was detected in eighty-one, Fifty-one of the patients (63%) had distal and 30 (37%) had proximal CCA. The overall sensitivity of EUS-FNA for the diagnosis of CCA was 73% and was significantly higher in distal compared to proximal CCA (81% *vs* 59%, respectively; $P = 0.04$). Furthermore, a retrospective analysis of 342 patients who underwent EUS-FNA after presenting with biliary stricture and obstructive jaundice[43] showed an overall 92.4% accuracy of EUS-FNA for diagnosing malignancy with 91.5% sensitivity and 80.9% negative predictive value. These studies and others demonstrate the higher sensitivity of EUS-FNA in distal biliary stricture. Moreover, EUS-FNA appears equivalent to ERCP sampling for biliary tumors and indeterminate strictures[41] and may provide a diagnosis of malignancy when ERCP sampling is indeterminate[44]. Moreover, EUS-FNA can have a role in diagnosing other lesions that may mimic cholangiocarcinoma and present either as a mass or with obstructive jaundice. Such lesions as epithelial *vs* nonepithelial tumors, neuroendocrine tumors, lymphoma, and metastasis from other primaries[45,46].

Endoscopic ultrasound-guided biliary access/drainage

ERCP is currently the standard of care for biliary drainage, however the failed cannulation rates ranges 3% to 5% in experienced hands. EUS-guided biliary drainage includes EUS-guided choledochoduodenostomy[47], hepaticogastrostomy[48], and EUS-guided transpapillary rendezvous biliary drainage[49]. The procedure technique has been described as follows[50]: the linear-array EUS scope is placed against the cardia or lesser curve of the stomach in a patient with dilated left intrahepatic biliary tree for hepaticogastrostomy or against the bulb of the duodenum for choledochoduodenostomy. The dilated bile duct or left intrahepatic duct which appears as hyperechoic structure running alongside the portal venous system without Doppler flow signals is then identified and punctured using a 19-guage or 22-guage needle. The stylet is then removed followed by contrast injection to visualize the biliary tree under fluoroscopy. A 0.035" or 0.021" guidewire is subsequently passed via the FNA needle into the bile duct or dilated intrahepatic duct. The needle knife is then used to make an incision of the gastric or duodenal wall under EUS guidance for preparation of dilation of the transmural tract. Dilation can be performed using 4.5F to 5F ERCP cannula, 4-mm or 6-mm dilating biliary balloon. A plastic biliary stent or self-expandable fully covered metal stent can then be placed[51,52]. In a large multicenter, nonrandomized retrospective study of 240 patients who underwent EUS-guided bile duct access and drainage[53], success was achieved in 87% of the cases. Similarly, in extrahepatic and intrahepatic approaches, the success rate was 84.3% *vs* 90.4%; respectively.

Gallbladder lesions

EUS-FNA has gained momentum in sampling gallbladder masses for diagnostic and staging purposes with accuracy reaching 100% in early stages. Sadamoto *et al*[54] reported EUS accuracy of 100% for in situ tumors (Tis), 76% for T1, 85% for T2, and 93% for T3 and T4 lesions. In one series, EUS-FNA provided accurate diagnosis of six patients with obstructive jaundice (five with gallbladder adenocarcinomas) where CT scans mostly failed to detect the causing lesions[55]. Jacobson *et al*[56] described similar findings in four out of five patients diagnosed with adenocarcinoma of the gallbladder. Meara *et al*[57] reported sensitivity of 80% and specificity of 100% in diagnosing gallbladder wall lesions.

EUS and transabdominal US are usually viewed as good tools to evaluate gallbladder polyps with superior sensitivities for EUS 97% *vs* transabdominal US 71% in one study[58]. Diagnostic distinction between malignant and non-malignant polyps for the purpose of staging and determining next steps management, remains mostly dependent on the ultrasonographic features of the polyps rather than tissue sampling[54]. No reports of the use of EUS-FNA in approaching gallbladder polyps were found. Endoscopic ultrasound-guided transmural gallbladder drainage with placement of self-expandable stent has

Table 2 Summary of the sensitivity, specificity and diagnostic accuracy of endoscopic ultrasound-guided fine needle aspiration in the diagnosis of focal hepatic, gallbladder and biliary tract lesions

Study, year, number	Sensitivity (%)	Specificity (%)	Diagnostic accuracy (%)
Focal malignant liver lesions			
DeWitt *et al*[4], 2003, *n* = 77	82-94	-	-
Hollerbach *et al*[11], 2003, *n* = 44	94	100	-
Singh *et al*[16], 2007, *n* = 17	89	100	94
	CT (71)	67	69
	MR (86)	100	92
Prachayakul *et al*[19], 2012, *n* = 14	78.5	-	-
Malignant biliary tract and gallbladder lesions			
Garrow *et al*[35], 2007, 36 studies, *n* = 3532	78	84	90
Nayar *et al*[36], 2011, *n* = 32	52	100	68
Fritscher-Ravens *et al*[37], 2004, *n* = 44	89	100	91
Weilert *et al*[41], 2014, *n* = 51	94	100	94
	ERCP brushing (50)	100	53
Mohamadnejad *et al*[42], 2011, *n* = 228	73	-	-
	ERCP brushing (27)		-
Tummala *et al*[43], 2013, *n* = 342	91.5	-	92.4
Meara *et al*[57], 2006, *n* = 53	80	100	-
	ERCP brushing (13)	75	-

CT: Computed tomography; MR: Magnetic resonance; ERCP: Endoscopic retrograde cholangiopancreaticography.

been reported and is technically successful for the management of acute cholecystitis in high risk patients[59-61]. Table 2 summarizes the sensitivity, specificity and diagnostic accuracy in the reported studies.

Ampullary tumors

EUS-FNA can provide an excellent diagnostic accuracy in distinguishing between benign and malignant ampullary tumors in comparison to surface biopsy with duodenoscopy, and/or intra-ampullary biopsy, and/or brush cytology with ERCP, and/or intra-ampullary biopsy after endoscopic sphincterotomy (100% *vs* 70%)[62]. Furthermore, the diagnostic accuracy of EUS-FNA for ampullary tumors supersedes that without EUS-FNA. In a retrospective study by Roberts *et al*[63], rates of diagnostic accuracy in high-grade dysplasia, low-grade dysplasia, and adenocarcinoma were 20%, 72%, and 96%, respectively, in the non-EUS group, and 50%, 93%, and 100%, respectively, in the EUS group.

ENDOSCOPIC ULTRASOUND AND RAPID ON-SITE CYTOLOGY EVALUATION

The diagnostic accuracy of EUS-FNA is dependent on how the sample is processed after acquisition. The presence of a rapid on-site cytology evaluation (ROSE) by a cytopathologist in the vicinity where the sample is obtained has been shown to improve the diagnostic yield of the procedure[64]. ROSE may allow a less number of needle passes and ensure adequacy of the sample obtained by onsite staining prior to completion of procedure. In general, the diagnostic yield of EUS-FNA with ROSE in most studies exceeds 90%. Meara *et al*[57] reported on 53 cases undergone EUS-FNA from 46 bile duct and seven gallbladder lesions where ROSE was available. All cases initially diagnosed as suspicious/malignant were confirmed on the final cytological interpretation. The specificity for EUS-FNA was 100% with sensitivity rates of 80% and 87% from clinically suspected malignancies of gallbladder and biliary tract, respectively. A retrospective study by Jhala *et al*[65] provided on-site diagnosis of malignancy on 485 EUS-FNA of the pancreas (*n* = 305), lymph nodes (*n* = 91), biliary tree (*n* = 47), liver (*n* = 15), gastrointestinal tract (*n* = 19), and adrenal gland (*n* = 8). A significantly higher degree of concordance was noted for unequivocal diagnosis of malignancy *vs* no malignancy (98.9% *vs* 67.2%) between on-site and final cytologic diagnosis. These studies have demonstrated ROSE by cytopathologist and interpretation significantly improves the diagnostic yield of EUS-FNA.

COMPLICATIONS OF ENDOSCOPIC ULTRASOUND-GUIDED FINE NEEDLE ASPIRATION OF HEPATIC AND BILE DUCT LESIONS

In a retrospective questionnaire sent to 130 EUS-FNA centers across the world[5]. 167 cases of EUS-FNA of the liver were reported by 21 centers. A complication was reported in 6 (4%) of the 167 cases including the following: death in 1 patient, bleeding (1), fever (2), and pain (2)[5]. EUS-guided liver biopsy appears to be safe and associated with no significant complications[2-4,66]. Several studies have reported no adverse events related to EUS-FNA of bile duct strictures, gallbladder and masses[41,42,56,57,67]. However, EUS-FNA of malignant biliary lesions was reported to have a risk of bleeding, infection, or pancreatitis in less than 2% of the cases[68]. Hemobilia was reported in 1.3% of patients who underwent EUS-FNA of malignant biliary stricture[42]. Bacteremia after EUS-FNA is rare. However, prophylactic antibiotics should be given prior and after EUS-FNA of biliary tract in patients with biliary obstruction. EUS-guided diagnostic abdominal paracentesis was not associated with any complication in one study[28]. Bile peritonitis has been reported after inadvertent biliary puncture during EUS-FNA[69]. Complications of EUS-guided biliary drainage included pneumoperitoneum 5%, bleeding 11%, bile leak/peritonitis 10%, and cholangitis 5%[53]. Needle track tumor seeding has been reported and is a risk after EUS-FNA of malignant biliary neoplasia[70,71]. EUS-FNA of malignant biliary stricture is considered a contraindication in patients eligible for liver transplantation. Cholecystitis and bile peri-

tonitis have been reported after EUS-FNA of gallbladder lesions[72]. Bleeding after EUS-FNA of solid tumor is rare and appears as an expanding extraluminal echopoor region adjacent to the sampled lesion[73].

LIMITATIONS OF ENDOSCOPIC ULTRASOUND-GUIDED FINE NEEDLE ASPIRATION OF HEPATIC AND BILE DUCT LESIONS

The head of pancreas and CBD are not visualized after Roux-en-Y surgery and Billroth Ⅱ surgery if the afferent limb is not intubated. Presence of vascular structures or collaterals in needle path may limit EUS-FNA of focal lesions. Because the right liver lobe is farther away from the probe, it is generally not seen except in small parts. The presence of pneumobilia, fatty infiltration, calcifications and extensive fibrosis may interfere with ultrasound beam and images. Endosonographer's experience, time consumed to image the liver and patient's body habitus are of critical importance to clearly identify and diagnose focal liver lesions. The miss rate for resectable pancreaticobiliary malignancy by EUS-FNA is rather small. Moreover, EUS and EUS-FNA may not be widely available and require an expertise with dedicated echosonographer in the field. With improving resolution and widespread use of EUS with dedicated formal training, small liver metastasis and other focal liver lesions are being increasingly detected. EUS does not use intravenous contrast to evaluate the nature of focal liver lesions and thus correlation with other cross-sectional imaging such as CT and/or MR is needed. However, the technology has dramatically improved. The use of color and power Doppler imaging, three-dimensional imaging, electronic scanning, tissue harmonic imaging, elastography, and recently contrast-enhanced images have improved the diagnostic capability. The depth of tumor infiltration and differentiation between infiltrating or exophytic lesions can now be assessed with greater accuracy[74-76].

CONCLUSION

Endoscopic ultrasound-guided fine needle aspiration of the liver, gallbladder and biliary tract is feasible and provides an excellent diagnostic accuracy. The presence of ROSE has increased the diagnostic yield. EUS-FNA is capable to differentiate between focal benign or malignant liver lesions. The widespread of EUS and increase formal training have enhanced the diagnostic and therapeutic armamentarium of EUS in hepatobiliary disorders. EUS-FNA should be considered as an adjunct to other cross-sectional imaging in the differentiation between benign and focal hepatobiliary disorders. EUS-guided interventions such as fine-needle injections, tumor ablative therapies and biliary drainage have increased the application of EUS and is considered as an adjunct to other modalities.

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Vitamin D and colon cancer

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Abstract

Calcitriol, 1α, 25-dihydroxyvitamin D_3 (1,25 $(OH)_2D_3$), the most active form of vitamin D, is a pleotropic hormone with a wide range of biological activities. Due to its ability to regulate calcium and phosphate metabolism, $1,25D_3$ plays a major role in bone health. In addition, $1,25D_3$ binds to the vitamin D receptor and thereby regulates the expression of a number of genes which control growth, differentiation and survival of cancer cells. In agreement, the levels of vitamin D_3 appear to be an essential determinant for the development and progression of colon cancer and supplementation with vitamin D_3 is effective in suppressing intestinal tumorigenesis in animal models. Vitamin D_3 has been estimated to lower the incidence of colorectal cancer by 50%, which is consistent with the inverse correlation between dietary vitamin D_3 intake or sunlight exposure and human colorectal cancer. Several studies confirmed that increasing vitamin D_3 lowers colon cancer incidence, reduces polyp recurrence, and that sufficient levels of vitamin D_3 are associated with better overall survival of colon cancer patients. Vitamin D regulates the homeostasis of intestinal epithelium by modulating the oncogenic Wnt signaling pathway and by inhibiting tumor-promoting inflammation. Both activities contribute to the ability of $1,25D_3$ to prevent the development and progression of colon cancer.

Key words: Colon cancer; Vitamin D; Wnt signaling; Inflammation; Chemoprevention

Core tip: Epidemiological studies suggest that deficiency of vitamin D increases the incidence of colon cancer and also has a negative impact on the survival of colon cancer patients. The ability of $1,25D_3$ to interfere with Wnt signaling and to ameliorate inflammation is likely to contribute to its anticancer activity.

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INTRODUCTION

The biologically active form of vitamin D_3,$1\alpha,25(OH)_2D_3$ ($1,25D_3$), is obtained by 25-hydroxylation of vitamin D_3 in the liver and 1α-hydroxylation in the kidney, liver or other tissues. Hydroxylation of $25(OH)D_3$ by CYP27B1 yields the hormonally active form $1,25(OH)_2D_3$, which is metabolized to less active metabolites by CYP24A1 (reviewed in[1]). While the levels of CYP21B1 have been shown to be reduced in some cancers, the levels of CYP24A1 are increased in cancer cells, which may contribute to the resistance of some tumors to $1,25D_3$[2].

$1,25D_3$ exerts most of its biological activity through binding to a specific vitamin D_3 receptor (VDR), a member of the nuclear receptor superfamily[1]. VDR binds to retinoid X receptor (RXR), and the VDR-RXR heterodimers bind to a vitamin D response element (VDRE), activating or repressing gene expression, which contribute to the anti-neoplastic activity of vitamin D. VDR associates with other transcription factors, such as SP1 and β-catenin[3] and thereby also regulates the expression of genes that do not harbor the consensus VDRE. A number of cancer cell lines, including colon cancer cell lines tested in our laboratory, display a limited response to vitamin D_3 *in vitro*[4] and the expression of VDR is

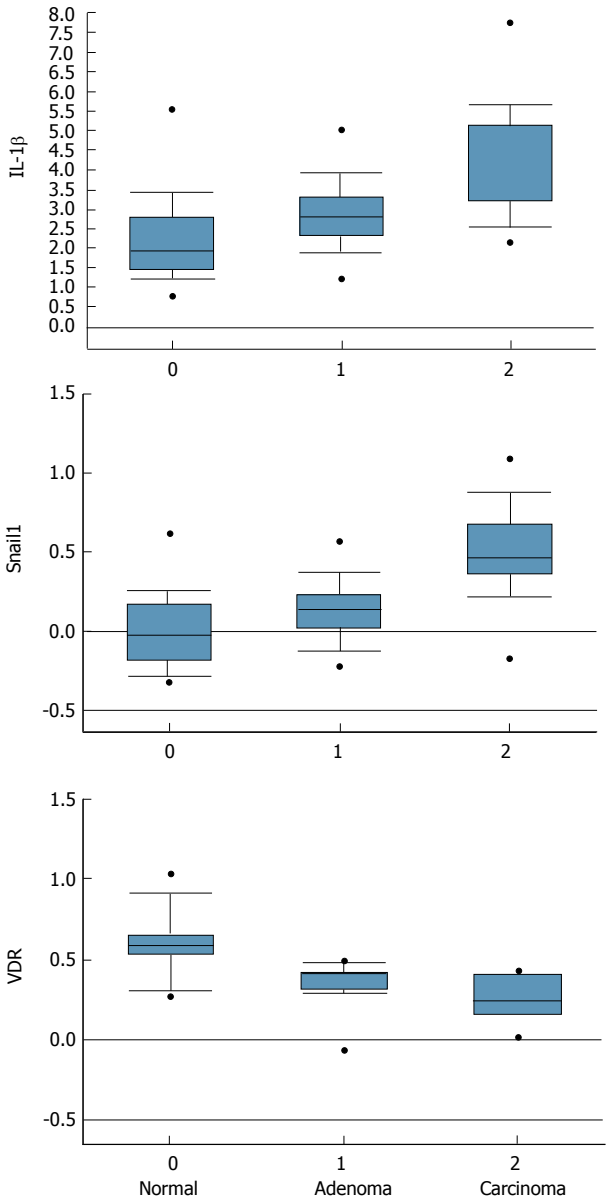

Figure 1 The expression levels of IL-1β and Snail are increased and the levels of vitamin D receptor decreased in colon cancer patients (Skrypzi-ack, *PLOS ONE* 2010[71]). VDR: Vitamin D receptor.

downregulated in late stages of colon cancer[5] (Figure 1), suggesting that vitamin D_3 may exert some of its biological activities in a VDR-independent manner, or that it targets cells in the tumor microenvironment. $VDR^{-/-}$ mice display hyper-proliferation and have elevated levels of c-myc in both skin and colon, and VDR suppresses c-myc expression *in vitro* and *in vivo* in the absence of $1,25D_3$[6]. However, $1,25D_3$ triggers association of VDR with c-myc and thereby promotes turnover of c-myc protein[6], indicating that vitamin D signaling suppresses transcription of c-myc and also inhibits c-myc stability. In addition to its ability to inhibit c-Myc, $1,25D_3$ induces the expression of its antagonist Mxd1/Mad1, suggesting that $1,25D_3$ can exert its chemopreventive activity through regulation of the c-myc/Mxd1 network[6].

The focus of this report is to discuss the role of vitamin D in colon cancer, however the beneficial effects of vitamin D have been noted in other malignancies. Reduced serum levels of vitamin D were found in stage IV melanoma patients and it has been shown that melanoma patients with low serum levels of vitamin D developed metastasis earlier than patients with high levels of vitamin D[7]. Similarly, chemopreventive activity of vitamin D has been observed in breast, ovarian, pancreatic and prostate cancer patients[8].

In addition to its chemopreventive activity, $1,25D_3$ or its analogues have been tested for their ability to improve the response to anticancer agents. Vitamin D and its derivatives have been shown to enhance the anticancer activity of 5FU, irinotecan and oxaliplatin both *in vitro* and *in vivo*[9,10]. Although the therapeutic use of $1,25D_3$ is restricted by its hypercalcemic activity, several $1,25D_3$ analogues that retain the antitumor activity while being devoid of hypercalcemic effects, are currently being tested in clinical trials for a variety of malignancies.

VITAMIN D AND COLON CANCER

Recent case-controlled studies have established that there is an inverse correlation between serum levels of vitamin D and the incidence of polyps and adenomas in the colon[11-13], consistent with the inverse correlation between dietary vitamin D_3 intake or sunlight exposure and human colorectal cancer[14-17]. This is significant because a large segment of the human population suffers from vitamin D_3 insufficiency or deficiency[18], which is particularly prevalent among colon cancer patients. Indeed, numerous studies have suggested that higher vitamin D_3 levels are associated with lower colon cancer incidence, reduced polyp recurrence and better overall survival of colon cancer patients[19-22].

Vitamin D and its analogues reduce the growth of colon cancer xenografts and inhibit tumorigenesis in several genetic models of intestinal cancer. In agreement, dietary initiation of colon cancer in rodents, a model of sporadic colon cancer, has been shown to be prevented by supplementation with vitamin D_3 and Ca[23,24].

Despite the established chemopreventive activity of vitamin D_3, its targets and the molecular basis for its antitumor activity remain poorly understood. $1,25D_3$ inhibits growth of tumor cells by inducing the expression of cyclin-dependent kinase inhibitors, such as p21, p27, and cystatin D, and by inhibiting the expression of pro-proliferative genes, including c-my and cyclin D1. In addition, $1,25D_3$ has been shown to upregulate miR-627, which targets the histone demethylase jumonji domain containing protein 1A, and thereby inhibits proliferation of colon cancer cells *in vitro* and *in vivo* through epigenetic regulation[25]. By increasing the expression of alkaline phosphatase, maltase, E-cadherin and cell adhesion proteins, vitamin D promotes differentiation. In a cell-type specific manner, vitamin D promotes apoptosis by regulating the expression of B-cell lymphoma 2 family members. Thus, due to its ability to affect multiple signaling pathways and to regulate many target genes, $1,25D_3$

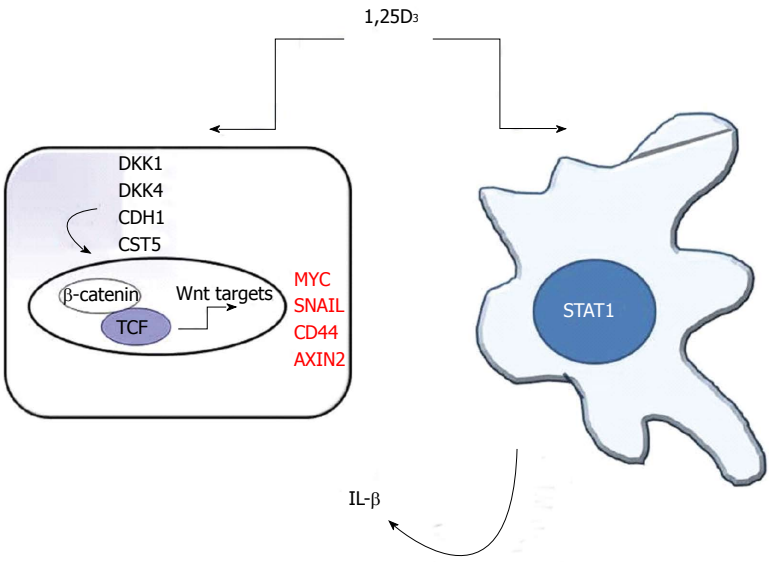

Figure 2 The multiple mechanisms whereby vitamin D inhibits Wnt signaling: 1,25D3 acts on both tumor cells and tumor-associated macrophages (and potentially on other stromal cells). In tumor cells, 1,25D3 promotes VDR/β-catenin binding and thus inhibits nuclear translocation of β-catenin. It also induces the expression of E-cadherin (CDH1), Dickkopf1 (DKK1), Dickkopf4 (DKK4) and cystatin 5 (CST5), antagonizing β-catenin/TCF transcriptional activity. As a result, the expression of several Wnt target genes, such as Snail, CD44, Myc, Axin2 (in red) is downregulated by 1,25D3. These activities require VDR expression in tumor cells. In addition, vitamin D also acts on cells in the tumor microenvironment. We demonstrated that 1,25D3 inhibits STAT1 activity in tumor-associated macrophages and prevents the release of IL-1β, which in a paracrine manner promotes Wnt signaling in cancer cells. 1,25D3 can thereby regulate Wnt signaling in tumor cells that do not respond directly to 1,25D3. VDR: Vitamin D receptor.

controls a variety of biological processes. Although 1,25D3 has also been shown in preclinical studies to inhibit invasiveness of tumor cells and to reduce their ability to metastasize, clinical trials suggest that while vitamin D is effective in early stages of cancer, it appears to have limited activity in advanced, aggressive malignancies.

Important mechanisms whereby 1,25D3 regulates the homeostasis of intestinal epithelium and exerts its anti-neoplastic activity is through its ability to interfere with Wnt/β-catenin signaling[3,26,27] and to inhibit inflammation. Because inflammation can fuel Wnt signaling in colon cancer cells, the two activities may be coupled, suggesting that 1,25D3 might exert chemopreventive activity by interrupting the link between inflammation and cancer. However, large clinical trials are required to firmly establish the preventive and therapeutic value of vitamin D in colon cancer. Such trials are complicated by the necessity of maintaining and monitoring vitamin D levels as well as clinical outcome in a large number of patients over a long period of time.

INHIBITION OF WNT SIGNALING BY VITAMIN D

The Wnt/β-catenin signaling pathway regulates the intracellular levels of β-catenin and controls the expression of β-catenin/TCF4 target genes. In normal cells, β-catenin is sequestered in a large cytoplasmic protein complex, called the β-catenin destruction box, which includes Axin and Apc and the GSK3β and CK1 kinases[28,29]. Due to mutations in the tumor suppressor Apc, or less frequently in Axin or β-catenin, the oncogenic Wnt/β-catenin signaling pathway is abnormally activated in over 90% of colon cancers[30].

The β-catenin destruction complex promotes β-catenin phosphorylation and its subsequent degradation. Wnt activation of its receptors, Frizzled and LRP5/6, inhibits the destruction complex and results in accumulation of β-catenin, both in the cytoplasm and in the nucleus, where it acts as a co-activator of LEF/TCF and regulates the expression of a variety of genes. Wnt/β-catenin signaling activates genes, such as c-myc and cyclin D and thereby promotes proliferation of tumor cells. Activation of Wnt signaling also induces the expression of COX2 and survivin which increases the survival of intestinal epithelial cells. Wnt signaling has been shown to promote transcription, protein stability and to regulate nuclear localization of Snail, a transcription factor that mediates epithelial mesenchymal transition[31,32]. In turn, Snail interacts with β-catenin and increases the expression of Wnt target genes[33]. We showed that inflammation-induced stabilization of Snail contributes to Wnt signaling in colon cancer cells and creates a positive feedback loop initiated, and propagated, by macrophage-derived IL-1β[34]. IL-1β was sufficient to increase the levels of Snail in colon cancer cells[35], and the levels of both IL1β and Snail are increased in colon cancer patients (Figure 1). Importantly, Snail1 and Slug (Snail2) have been shown to inhibit the expression of VDR and to inhibit the activity of 1,25D3[5,36-38]. Wnt-dependent stabilization of Snail is likely to contribute to reduced expression of VDR in colon cancer patients (Figure 1).

1,25D3 has been shown, in a VDR-dependent manner, to antagonize Wnt signaling through a variety of mechanisms. These include sequestration of β-catenin through a direct VDR/β-catenin interaction and induction of nuclear export of β-catenin. 1,25D3 also enhances the expression of DKK1, which is an endogenous inhibitor of Wnt signaling. Furthermore, cystatin D, whose expression is strongly upregulated by 1,25D3, inhibits Wnt signaling and the expression of its target genes, including Snail (Figure 2). Cystatin D inhibits migration and anchorage- independent growth of colon cancer cells and its silencing abrogates the anti-proliferative activity of 1,25D3 and increases the expression of c-Myc[39]. A comprehensive review of the mechanisms whereby vitamin D represses Wnt signaling has been published recently[40].

Wnt activity in primary human tumors is heterogeneous, and it has been demonstrated that its activity is

regulated by factors from the tumor microenvironment. Although loss of Apc occurs early in adenoma development in the colon, *in vivo* progression from micro-adenomas to macroscopic tumors in *Apc*$^{Min/+}$ mice is associated with further elevation of canonical Wnt signaling and increased expression of Wnt target genes[41]. This suggests that enhancement of Wnt signaling beyond a threshold level sufficient for tumor initiation may be required for tumor progression and metastatic spread. Often factors from the tumor microenvironment provide signals that regulate the extent of oncogenic signaling in tumor cells. We and others have demonstrated that tumor-associated macrophages promote Wnt signaling in colon cancer cells *via* IL-1β and TNF[34,42]. Fibroblasts have also been shown to enhance Wnt signaling through hepatocyte growth factor[43], confirming the role of inflammatory factors in Wnt signaling and in maintenance of cancer stem cells (see below). Leukotriene D4, which can be produced and secreted by stromal cells in the local tumor microenvironment, promotes the expression and nuclear translocation of β-catenin and thus enhances the growth of colon cancer cells[44]. Indeed, β-catenin translocation is often detected at the invasive front of tumors[45,46], consistent with the interpretation that stromal tissue at the invasion front provides signals to tumor cells that promote nuclear translocation of β-catenin and thus drive tumor progression. It is therefore likely that 1,25D$_3$ regulates Wnt signaling by targeting both the tumor microenvironment as well as the tumor cells themselves. Indeed, we have shown that vitamin D interrupts signaling between tumor cells and macrophages and thereby decreases the intensity of Wnt signaling in HCT116 colon cancer cells which are themselves unresponsive to direct effect of vitamin D[34]. We demonstrated that this mechanism involved 1,25D$_3$ inhibition of STAT1 activity in macrophages, blocking the release of IL-1 and thereby restoring the sensitivity of colon cancer cells to TRAIL-induced apoptosis[35]. This is in line with the concept that the tumor microenvironment represents an important target of chemopreventive and chemotherapeutic agents[47].

The ability of vitamin D to regulate Wnt signaling has been confirmed in animal models. Vitamin D and its analogues reduced the number of tumors in Apc$^{Min/+}$ mice[48], associated with decreased nuclear β-catenin and reduced expression of β-catenin target genes[49]. Likewise, dietary induction of colon tumors in mice, a model of sporadic colon cancer, accompanied by functional enrichment of Wnt signaling, is reversed by supplementation with vitamin D and Ca[24]. Apc$^{Min/+}$ mice lacking VDR have an increased number of aberrant crypt foci (ACF) and both ACFs and tumors in Apc$^{Min/+}$/VDR$^{-/-}$ mice display increased nuclear β-catenin and elevated expression of β-catenin/TCF target genes[50]. While the number of adenomas and carcinomas was not affected by the inactivation of VDR, tumors that developed in the Apc^{Min+}/VDR$^{-/-}$ mice were significantly larger, consistent with increased growth dues to enhanced Wnt signaling. We recently confirmed that while targeted inactivation of VDR in intestinal cells did not alter tumor multiplicity in Apc$^{Min/+}$ mice, inactivation of VDR in macrophages substantially reduced Apc$^{Min/+}$ tumors (submitted), confirming the important role of VDR signaling in the tumor microenvironment.

Consistent with these *in vitro* data and with studies in mice, dietary supplementation with 1,25D$_3$ decreased the levels of β-catenin and increased the expression of E-cadherin in normal mucosa of colon cancer patients[51].

ANTI-INFLAMMATORY PROPERTIES OF VITAMIN D

Chronic inflammation has been shown to predispose to development of tumors, a striking example being inflammatory bowel disease, which is associated with elevated risk of colon cancer[52]. Moreover, it appears that colon cancers that are not linked to inflammatory bowel disease are also driven by inflammation; it has been shown that regular use of NSAIDs lowers the mortality from sporadic colon cancer and inhibits adenomas in FAP patients, who inherit a mutation in the *Apc* gene[53]. The mechanisms whereby anti-inflammatory agents inhibit progression of tumors that are not associated with overt inflammation are not fully understood. However, it has been established that cancer and several other chronic diseases are associated with para-inflammation, a low grade inflammation that is coupled to a persistent activation of the DNA damage response[54] and the induction of DNA damage-induced soluble factors, including major pro-inflammatory cytokines, chemokines and growth factors. It is possible that anti-inflammatory agents exert their chemopreventive activity by ameliorating the pro-tumorigenic activity of para-inflammation that is associated with aging and that is observed in colon cancer patients.

Inflammatory bowel disease (IBD) is among the three most prevalent high risk conditions for colon cancer[52]. The risk for colorectal cancer increases with the duration and the extent of the disease, consistent with a direct connection between inflammation and colon cancer development. Patients with intestinal inflammatory conditions such as ulcerative colitis (UC) and Crohn's disease (CD) have a high incidence of vitamin D insufficiency and deficiency[55] and show reduced levels of VDR in intestinal epithelium[56]. Likewise, higher levels of vitamin D have been shown to lower the risk of Crohn's disease[57]. Overexpression of VDR in intestinal cells inhibits the colitis-associated increase in proinflammatory cytokines, such as TNF, IL-1 and CCL2, and protects mice from developing colitis[56]. Finally, a vitamin D analogue has been shown to inhibit colon carcinogensis in the azoxymethane/dextran sodium sulphate (AOM/DSS) model of ulcerative colitis[58], suggesting that VDR signaling may avert the conversion of the inflammatory stimuli into a tumor promoting signal.

VDR knockout mice exhibit a proinflammatory phenotype associated with increased NF-κB activity in intestine, consistent with the ability of VDR signaling to inhibit NF-κB activation[59]. TNF-α is a major proin-

flammatory cytokine that activates the NF-κB signaling pathway in tumor cells and thereby regulates their growth and survival. Human colon cancers are infiltrated by inflammatory cells which secrete a variety of proinflammatory factors, including TNF-α[60]. Likewise, polyps arising in $Apc^{\Delta 468}$ mice, a genetic model for intestinal cancer, showed infiltration with mast cells, and depletion of mast cells or anti-TNF-α treatment significantly suppressed polyposis in $Apc^{\Delta 468}$ mice[60]. Etanercept, a specific antagonist of TNF-α, also reduced the number and the size of tumors in the AOM/DSS model, confirming a role of TNF-α in inflammation-promoted intestinal tumorigenesis. More intriguing was the observation that inhibition of TNF-α blocks the accumulation of β-catenin mutations in intestinal cells, suggesting a mutagenic role of TNF-α[61]. Pharmacological inhibition of TNF-α by neutralizing TNF-α antibodies is very effective in alleviating inflammation in IBD patients[62] and inhibitors of TNF-α have also been tested as potential agents for the treatment of colon cancer. Unfortunately, TNF-α inhibitors have been linked to a broad range of infections and to the development of lymphomas and skin and lung cancer, limiting their clinical utility.

An alternative approach to targeting TNF/NF-κB-mediated inflammation and interrupting the link between inflammation and cancer may be offered by vitamin D. $1,25D_3$ inhibits the interaction of peripheral blood mononuclear cells and colon cancer cells and inhibits the production of TNF[63] and blocks NF-κB signaling, a major TNF signaling pathway. VDR physically interacts with IKKβ[59] and vitamin D downregulates the expression of NFκB target genes, such as Puma[56], which play a major role in the survival of cancer cells. In addition, $1,25D_3$ has been shown to downregulate the expression of Toll-like receptors 2 and 4 (TLR2 and TLR4) on human monocytes, resulting in hyporesponsiveness to TLR activating ligands[64,65]. Inhibition of TLR signaling by vitamin D_3 has been suggested to reduce AOM/DSS- induced colon cancer[66], pointing to a convergence of the chemopreventive and anti-inflammatory properties of vitamin D_3.

NF-κB is not the only oncogenic signaling pathway activated in tumor cells by inflammatory factors. We have shown that TNF enhances Wnt signaling in β-catenin mutant colon cancer cells[34], and established that macrophage-derived factors activate Wnt signaling in colon cancer cells through NF-κB signaling[42]. Oguma *et al*[67] demonstrated that TNF-β promotes Wnt signaling also in gastric cancer cells, which was independent of NF-κB in this tissue.

The HCT116 colon cancer cells have a functional VDR, but do not respond to $1,25D_3$ treatment with growth arrest, apoptosis or differentiation. However, we demonstrated that in the presence of macrophages, $1,25D_3$ reduced Wnt signaling in these seemingly vitamin D unresponsive cells by interrupting signaling between tumor cells and macrophages. $1,25D_3$ inhibits STAT1 activity and prevents tumor cell-induced release of IL1 from macrophages and thereby prevents inflammation-induced Wnt signaling in colon cancer cells[34] (Figure 2). Accordingly, $1,25D_3$ inhibits the ability of macrophages to increase proliferation and survival of colon cancer cells. Among genes that were repressed by $1,25D_3$ in tumor cells in a macrophage-dependent manner were cyclin D1 and c-myc, consistent with the finding that $1,25D_3$ prevented macrophage-induced clonogenic growth of HCT116 cells. Therefore, $1,25D_3$ can exert its tumor-preventive activity by normalizing the tumor microenvironment, and it can inhibit inflammation through a variety of mechanisms.

Diet-induced obesity, a risk factor for colon cancer, is also associated with increased expression of TNF-β in the intestine. In this settings, TNF-β has also been shown to be coupled to inactivation of GSK3-β and increased expression of β-catenin and c-myc, suggesting that obesity increases the risk of colorectal cancer by promoting inflammation[68]. Indeed, western style diet (WSD), sufficient to initiate intestinal tumorigenesis in mice[24], has been shown to trigger an inflammatory response in mice, accompanied by the accumulation of macrophages in intestinal mucosa and increased levels of circulating proinflammatory cytokines, including IL-1β, CCL5 and CCL2[69]. Importantly, dietary supplementation with vitamin D and Ca prevents WSD-induced increases in inflammatory markers and inhibits intestinal tumorigenesis[24,69]. Dietary supplementation with $1,25D_3$ reduced markers of inflammation, including C-reactive protein (CRP), TNF, IL-1β, IL-6 and IL-8 also in colon cancer patients[70], strongly suggesting that $1,25D_3$ protects from colon cancer, at least in part, by decreasing inflammation.

CONCLUSION

Calcitriol, the most active form of vitamin D_3, acts as a potent steroid hormone that binds to VDR and thereby alters the expression of a variety of genes that regulate growth, differentiation and survival of epithelial cells. Epidemiological studies suggest that deficiency of vitamin D increases the incidence of colon cancer and also has a negative impact on the survival of colon cancer patients. The ability of $1,25D_3$ to interfere with Wnt signaling and to ameliorate inflammation is likely to contribute to its anticancer activity. The optimal form and adequate concentration of vitamin D that have cancer preventive activity should be established, and randomized clinical trials are needed to confirm that $1,25D_3$ alone, or in combination with other cytotoxic agents, offers therapeutic benefits.

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MINIREVIEWS

Neoadjuvant chemoradiotherapy for locally advanced rectal cancer: The debate continues

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Abstract

Rectal carcinoma represents the 30% of all colorectal cancers, with about 40000 new cases/years. In the past two decades, the management of rectal cancer has made important progress, highlighting the main role of a multimodality strategy approach, combining surgery, radiation therapy and chemotherapy. Nowadays, surgery remains the primary treatment and neo-adjuvant chemoradiotherapy, based on fluoropyrimidine (5-FU) continuous infusion, is considered the standard in locally advanced rectal carcinoma. The aim is to reduce the incidence of local recurrence and to perform a conservative surgery. To improve these purposes different drugs combination have been tested in the neo-adjuvant setting. At American Society of Clinical Oncology 2014 an important abstract was presented focusing on the role of adding oxaliplatin to concomitant treatment, in patients with locally advanced rectal carcinoma. Rodel *et al* reported on the CAO/ARO/AIO-04 randomized phase Ⅲ trial that compared standard treatment with 5-FU and radiation therapy, to oxaliplatin plus 5-FU in association with radiation therapy. The addition of oxaliplatin to the neo-adjuvant treatment has been shown to improve disease-free survival from 71.2% to 75.9% (P = 0.03). This editorial was planned to clarify the optimal treatment in patients with locally advanced rectal cancer, considering the results from CAO/ARO/AIO-04 study.

Key words: Chemoradiotherapy; Rectal cancer; Locally advanced disease; Neoadjuvant; Debate

Core tip: This editorial was planned to clarify the optimal treatment in patients with locally advanced rectal cancer, considering the results from CAO/ARO/AIO-04 trial.

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In locally advanced rectal cancer, significant progress has been made over the past few decades for improving loco-regional control: total mesorectal excision standardization, radiotherapy dose fractionation, correct timing of treatment modalities, integration of diverse chemotherapy agent into the chemoradiotherapy regimes[1]. The German Rectal Cancer Study Group addressed the last of those controversies, and in a multicentre randomised phase Ⅲ study, the CAO/ARO/AIO-04 trial, compared oxaliplatin (OXP) and fluoropyrimidine (5-FU) in combination with radiation *vs* 5-FU with radiation as neoadjuvant long-course treatment[2].

The essential function of OXP with 5-FU has been demonstrated in colon carcinoma; survival rates, both overall and disease-free, were significantly improved in

patients who received OXP and 5-FU as adjuvant treatment[3]. Considering these results, several groups, despite the absence of a randomised comparison in neoadjuvant setting for rectal cancer, designed phase Ⅲ studies to test the standard 5-FU-based neoadjuvant treatment *vs* analogous but new schedule where the monochemotherapy was replaced by a combination of 5-FU and OXP[2,4-6]. This drugs-radiation combination have failed in increase primary tumor response in STAR-01 study[4], ACCORD 12/0405-Prodige-2 study[5] and NSABP-R04 study[6], whereas the results of the CAO/ARO/AIO-04 study are intriguing[2]. The second arm in the CAO/ARO/AIO-04 study represented a "experimental" schedule, which used a continuous venous infusion of 5-FU 200 mg/m^2 and a 2-h OXP infusion 50 mg/m^2. The 5-FU was delivered during days 1-14 and 22-35, whereas the OXP was delivered days 1, 8, 22 and 29. The primary end-point was disease-free survival (DFS) at 3 years, with acute toxicity, compliance and histopathological response as secondary endpoints. A total of 1265 patients were randomly enrolled, 637 were assigned to control arm and 628 to experimental arm. Acute treatment-related toxicity was similar in the two arms except for 7% grade 3-4 sensory neuropathy events in the OXP-5-FU arm - an obvious expected result of the pharmacokinetics of OXP adsorption. The compliance, defined as full prescribed dose of chemotherapy and full dose of radiotherapy was comparable. Pathological complete response (pCR) was gained in 17% of patients on OXP-5-FU *vs* 13% on 5-FU (P value = 0.038). With a median follow-up of 50 mo, the 3-years DFS rate was 75.9% in the OXP-5-FU arm *vs* 71.2% in the control (P = 0.03).

What deductions can we reach from this study? A key observation is that compliance to "experimental" schedule is high and successful disease control is achieved. The other published randomised trials - it is important to note that different OXP and fluoropirimidine schedules were used in STAR-01 study[4], ACCORD 12/0405-Prodige-2 study[5] and NSABP-R04 study[6] - used a continuous infusion of chemotherapy during radiation therapy. Therefore, the one week gap from the conventional administration of 5-FU and OXP is a valid option, with a more tolerable profile. Achieve pCR is a good end-point in rectal cancer, and could be used as prognostic factor - pCR is correlated to excellent long-term prognosis - to recommend a "wait and see" approach, without adjuvant chemotherapy[7].

So where do we go from here? There is a considerable agreement in the administration of neoadjuvant chemotherapy plus radiotherapy for the treatment of locally advanced rectal cancer. In many of the trials undertaken and those that are ongoing, 5-FU-based chemoradiotherapy represents the cornerstone, due to fluoropyrimidine well-established potentiating effect with radiation. OXP should be added to influence the tumour cell sensitivity, resulting in a higher rate of down-staging, delineating different subgroups of patients and changing the risk of recurrences.

Although the addition of oxaliplatin to standard neoadjuvant regimen appears tolerable, it is true that the real benefit of OXP-5-FU remains unclear. The CAO/ARO/AIO-04 study has confirmed a DFS improvement; do we therefore conclude that OXP-5-FU combination provides indication of survival benefit in locally advanced rectal cancer? There are not randomized studies that have shown a statistical benefit from adding OXP to standard neoadjuvant chemoradiotherapy. Certainly, the results of the CAO/ARO/AIO-04 study represent a step in the right direction: it demonstrates the feasibility of neoadjuvant-intensified chemoradiotherapy in a multidisciplinary treatment approach setting for rectal cancer.

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RETROSPECTIVE STUDY

Incidental gall bladder cancers: Are they truly incidental?

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Abstract

AIM: To seek and analyze features suggestive of gallbladder cancer (GBC) on preoperative imaging and intraoperative findings in patients diagnosed as having incidental GBC (IGBC).

METHODS: The study was conducted on 79 patients of IGBC managed in our department over a 10-year period (2003-2012). Review of preoperative imaging and operative notes was done to ascertain any suspicion of malignancy-in-retrospect.

RESULTS: Of the 79 patients, Ultrasound abdomen showed diffuse thickening, not suspicious of malignancy in 5 patients, and diffuse suspicious thickening was seen in 4 patients. Focal thickening suspicious of malignancy was present in 24 patients. Preoperative computed tomography/magnetic resonance imaging was done in 9 patients for suspicion of malignancy. In 5 patients, difficult Cholecystectomy was encountered due to dense/inflammatory adhesions. Intraoperative findings showed focal thickening of the gallbladder and a gallbladder mass in 9 and 17 patients respectively. On overall analysis, 37 patients had preoperative imaging or intraoperative findings suggestive of malignancy, which was either a missed GBC or an unsuspected/unexpected GBC. In 42 (53.2%) patients, there was no evidence suggestive of malignancy and was an unanticipated diagnosis.

CONCLUSION: Our study highlights a potential and not-so-rare pitfall of Laparoscopic Cholecystectomy. A greater awareness of this clinical entity along with a high index of suspicion and a low threshold for conversion to open procedure, especially in endemic areas may avert avoidable patient morbidity and mortality.

Key words: Incidental gallbladder cancer; Preoperative detection; Imaging

Core tip: The true incidence of incidental gallbladder cancer (IGBC) in literature appears skewed as the preoperative and intraoperative clues towards malignancy may be missed. We aimed to seek and analyze features suggestive of GBC on preoperative imaging and intraoperative findings in patients diagnosed as having IGBC. On overall analysis, 37 patients had preoperative imaging or intraoperative findings suggestive of malignancy, which was either a missed GBC or an unsuspected/unexpected GBC. A greater awareness of this clinical entity along with a high index of suspicion and a low threshold for conversion to open procedure, may avert avoidable patient morbidity and mortality.

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Table 1 Preoperative radiology

Radiology	*n* (%)
Ultrasonogram - diffuse suspicious thickening	4 (5.1)
Ultrasonogram - diffuse thickening not suspicious of malignancy	5 (6.3)
Ultrasonogram - focal Thickening	24 (30.4)
CT/MRI - suspicious lesion	9 (11.4)
Non specific	42 (53.2)

CT: Computed tomography; MRI: Magnetic resonance imaging.

Table 2 Intraoperative findings

Intraoperative findings	*n* (%)
Focal thickening of gallbladder	9 (11.4)
Gallbladder mass	17 (21.5)
Difficult cholecystectomy (dense/inflammatory adhesions)	5 (6.3)
Uneventful	48 (60.1)
Conversion to open cholecystectomy	5 (6.3)

INTRODUCTION

Laparoscopic cholecystectomy (LC) has been the gold standard treatment for gallstone disease for over two decades. LC performed for gallstone disease may rarely result in a diagnosis of unexpected gallbladder cancer[1-3]. Incidental gallbladder cancer (IGBC) may be defined as a malignancy detected only on histopathological examination without prior pre-operative or intra-operative suspicion of malignancy[4]. The incidence of gallbladder cancer diagnosed during or after LC is 0.2%-2.85%. With ever increasing numbers of laparoscopic cholecystectomies being performed worldwide, an incidental diagnosis of gallbladder carcinoma is also becoming more frequent[1,2,4,5-9]. In patients with IGBC, many will have residual disease, and their survival may be worse as compared to those who have undergone a radical procedure as the index surgery; thus a preoperative diagnosis of GBC is imperative[10-12]. The aim of the study was to seek and analyze features suggestive of GBC on preoperative imaging and intraoperative findings in patients diagnosed as having IGBC.

MATERIALS AND METHODS

An analysis from a prospectively collected database of patients admitted in our department with a diagnosis of IGBC following a Laparoscopic/Laparoscopy converted to open cholecystectomy for gallstone disease between Jan 2003 and Dec 2012 was done. The group consisted of patients who had undergone the index cholecystectomy either in our unit or elsewhere. All histological reports and slides were reviewed and verified to confirm the presence of gallbladder cancer. Over a 10-year period (2003-2012), 79 patients were operated for IGBC. Their preoperative radiological findings and operative notes were reviewed to asceratain any suspicion of malignancy-in-retrospect.

RESULTS

Diffuse thickening not suspicious of malignancy and diffuse suspicious thickening of the gallbladder were seen in 5 and 4 patients respectively on ultrasound abdomen. Suspicious focal thickening was observed in 24 patients. 53.2% of the patients had unremarkable preoperative imaging (Table 1). In 5 patients, a difficult Cholecystectomy was encountered due to dense/inflammatory adhesions. Intraoperative findings of a focal thickening of the gallbladder and a gallbladder mass were observed in 9 and 17 patients respectively (Table 2). Five patients needed conversion to open cholecystectomy, an incidence of 6.3% which was far higher than our unit's conversion rate from laparoscopic to open cholecystectomy of 1.3%. On overall analysis, 37 patients had preoperative imaging or intraoperative findings suggestive of malignancy, which was either a missed GBC or an unsuspected/unexpected GBC. On the contrary in 42 (53.2%) patients, there was no pre/peroperative evidence suggestive of malignancy and IGBC was a histological surprise (Table 3).

DISCUSSION

Stage matched outcomes following surgery for IGBC may be significantly worse than those operated with an initial diagnosis GBC; therefore, a preoperative diagnosis is imperative and helps decrease long term morbidity and mortality[2,4-13].

Due to its myriad presentations, the radiological and clinical features of gallstone disease may mask GBC; making a preoperative diagnosis of GBC in these patients difficult. Both entities may have similar clinical features such as those suggestive of acute or chronic cholecystitis and radiological findings such as thickening of the gallbladder wall and/or polyps[2,10,14,15]. In our study, 46.8% of the patients had subtle signs suggestive of a pathology other than gallstone disease, which were overlooked during the index surgery. Preoperative identification of patients at a higher risk of IGBC like those with gallbladder polyps or a mass on imaging, might forewarn a surgeon, and allow for the performance of an adequate R0 resection at the initial procedure or a possible referral to a center with expertise in liver surgery[1,2,4]. Intraoperatively, a difficult cholecystectomy, should raise the suspicion of an IGBC especially in endemic areas[8,14,15]. Operative management should be appropriately altered based on intraoperative findings and a liberal application of frozen section examination. In our series, there was a significantly higher rate of conversion to open cholecystectomy in patients with IGBC who had their index operation at our institute as compared to our standard conversion rate.

A combination of clinical and radiological factors combined with a liberal application of intraoperative frozen section examination can help guide the surgeon towards a structured and rationalized management of IGBC. Differentiating IGBC from gallstone disease is a

Table 3 Preoperative suspicion of gallbladder cancer

Incidental gallbladder cancer - pre/intraoperative picture	*n* (%)
Suggestive of malignancy	37 (46.8)
Unanticipated diagnosis	42 (53.2)

diagnostic conundrum. Making this distinction preoperatively or intraoperatively may be difficult and a definitive diagnosis still necessitates a histopathological examination. An accurate preoperative diagnosis requires an integrated review of clinical and characteristic radiological features, the presence of which may help guide surgery and prevent avoidable morbidity in selected cases.

In conclusion, our results showcase a potential and not-uncommon hazard of Laparoscopic Cholecystectomy. A better understanding along with a heightened suspicion and a low threshold for conversion to an open procedure particularly in endemic areas will help avoid preventable patient morbidity and mortality.

COMMENTS

Background

Incidental Gallbladder cancer (IGBC) is defined as cancer detected for the first time on histopathological examination with no pre-operative or intra-operative suspicion of malignancy. With the wide acceptance of laparoscopic cholecystectomy, an incidental diagnosis of gallbladder carcinoma is becoming more frequent.

Research frontiers

However, the true incidence of IGBC in literature appears skewed as the pre-operative and intraoperative clues towards malignancy may be missed. The authors aimed to seek and analyze features suggestive of GBC on preoperative imaging and intraoperative findings in patients diagnosed as having IGBC.

Innovations and breakthroughs

On overall analysis, 37 patients had preoperative imaging or intraoperative findings suggestive of malignancy, which was either a missed GBC or an unsuspected/unexpected GBC. In 42 (53.2%) patients, there was no evidence suggestive of malignancy and was an unanticipated diagnosis.

Applications

This study highlights a potential and not-so-rare pitfall of Laparoscopic Cholecystectomy. A greater awareness of this clinical entity along with a high index of suspicion and a low threshold for conversion to open procedure, especially in endemic areas may avert avoidable patient morbidity and mortality.

Terminology

IGBC is defined as cancer detected for the first time on histopathological examination with no pre-operative or intra-operative suspicion of malignancy.

Peer review

The authors have written an interesting paper regarding the incidental gall bladder cancers. Overall the paper is well organized and quite educative giving new information. This study highlights a potential and not-so-rare pitfall of Laparoscopic Cholecystectomy and suggests that it is necessary a greater awareness of this clinical entity and so could prevent avoidable patient morbidity and mortality.

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OBSERVATIONAL STUDY

TT genotype of *GNAS1 T393C* polymorphism predicts better outcome of advanced non-small cell lung cancer patients

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Abstract

AIM: To evaluate the potential prognostic value of *GNAS1 T393C* polymorphism in advanced non-small cell lung cancer.

METHODS: We extracted genomic DNA from the peripheral blood leucocytes of 94 patients with advanced non-small cell lung cancer. Quantitative real-time polymerase chain reaction was used to determine the allelic discrimination. The correlation between genotype and overall survival was evaluated using the multivariate analysis and Kaplan-Meier approach.

RESULTS: Thirty-eight out of 94 (40%) patients displayed a TT genotype, 29 out of 94 (31%) a CT genotype and 27 out of 94 (29%) a CC genotype. The median survival of TT (25 mo) genotype carriers was longer than CT (12 mo) or CC (8 mo) genotype carriers. The favorable TT genotype predicted better overall survival (OS) (2-year OS: 48%; P =0.01) compared with CT (2-year OS: 18%) or CC (2-year OS: 15%) genotype. However, dichotomization between C-genotypes (CC + CT) and T-genotypes (TT) revealed significantly lower survival rates (2-year OS: 16%; P = 0.01) for C allele carriers.

CONCLUSION: Our data provided strong evidence that the *GNAS1 T393C* genetic polymorphism influenced the prognosis in advanced non-small lung cancer with a worse outcome for C allele carriers.

Key words: *GNAS1*; Polymorphism; Advanced non-small cell lung cancer; Prognosis

Core tip: We genotyped *GNAS1 T393C* single nucleotide polymorphism in a homogenous (Han) study population of patients to evaluate the effect of this polymorphism on survival in non-small cell lung cancer (NSCLC). Our study indicated that the *GNAS1 T393C* polymorphism affected the overall survival in advanced NSCLC with a worse outcome for C allele carriers.

Gong HY, Hu WG, Wang XL, Zhu F, Song QB. TT genotype of *GNAS1 T393C* polymorphism predicts better outcome of advanced non-small cell lung cancer patients. *World J Gastrointest Oncol* 2014; 6(12): 444-449 Available from: URL: http://www.wjgnet.com/1948-5204/full/v6/i12/444.htm DOI: http://dx.doi.org/10.4251/wjgo.v6.i12.444

INTRODUCTION

The incidence of lung cancer has increased substantially over the past ten years[1]. Non-small cell lung cancer (NSCLC) constitutes about 85% of all lung cancer cases[2] with only 16.6% being able to live 5 years or more

after diagnosis[3]. To date, the most feasible treatment for advanced NSCLC patients is the platinum-based combination chemotherapy and it turns out to be associated with better overall survival rates[4]. Tumor-node-metastasis stage normally correlates with the clinical outcome of a large population of patients, but patients with similar clinical characteristics have different outcomes, which may be affected by their individual genes. The identification of patients with high-risk lung cancer could thus help to set up novel treatment strategies and could theoretically improve the outcome of anti-cancer therapy. Therefore, it is desirable to characterize more reliable and accurate molecular markers to identify more aggressive lung cancer phenotypes in order to individually tailor the therapy.

Actually, previous studies have implied that biomarkers could help define the subgroups of patients. However, there is no standard way to immunohistochemically detect these biomarkers, which prevents their application as prognostic factors. Nowadays, people choose to study single nucleotide polymorphisms (SNPs) as prognostic markers because these SNPs can be easily evaluated using patients' blood samples, which can avoid issues such as the availability and the quality of materials. One typical example is the *GNAS1 T393C* polymorphism.

The *GNAS1* gene has been mapped to chromosome 20q13 and contains 13 exons. The *GNAS1 T393C* polymorphism is located in exon 5, which encodes the α-subunit of the stimulatory G protein, namely Gαs. Somatic mutations of *GNAS1* have been reported to be involved in the etiology of McCune-Albright syndrome and sporadic, isolated endocrine tumors[5-7], suggesting that *GNAS1* could participate in cancer initiation and progression. What's more, previous studies have demonstrated that the *T393C* polymorphism was significantly correlated with the prognosis of patients with various cancers, such as breast carcinoma, squamous cell carcinoma of the larynx, bladder cancer, cholangiocarcinoma, colorectal cancer, clear cell renal carcinoma, and cancers of the oropharynx and hypopharynx[8-20].

In this study, we genotyped the *T393C SNP* in a Han population to evaluate the effect of this polymorphism on lung cancer prognosis. Our purpose was to determine whether the common *GNAS1 T393C* polymorphism can be used as a predictive factor for survival in NSCLC patients.

MATERIALS AND METHODS

Patients and clinical samples

Two milliliters of peripheral blood samples were collected from patients diagnosed with advanced NSCLC pathologically before any antineoplastic treatment at Renmin Hospital of Wuhan University (China) between March 2010 and March 2012. Patients were chosen based on the following criteria: (1) histologically confirmed UICC (2009) stage ⅢB or Ⅳ NSCLC; (2) Eastern Cooperative Oncology Group performance status (PS) score of 2 or less; and (3) life expectancy of more than 3 mo. Patients were not included if they had received any anti-tumor therapy previously. All patients were asked to sign the informed consent before they were included in the database. The study cohort (94 patients; for clinicopathological data, Table 1) composed exclusively of patients with a meticulously complete follow-up record. This study was performed following the guidelines of the local research ethics committee.

DNA extraction and genotyping

Genomic DNA was isolated from whole blood samples using the QIAamp kit (Qiagen, Germany). *T393C* SNP (dbSNP rs7121) was amplified by polymerase chain reaction (PCR) with the following primers: 5'-CAGCCCACATTAGGGAGCATAT-3' (forward) and 5'-TAATCCCTGCCTATGCTCACGA-3' (reverse). After denaturation at 95 ℃, 50 cycles of DNA amplification was done using $(NH4)_2SO_4$ containing buffer (Bioron, Germany) at 95 ℃ for 60 s, 60 ℃ for 30 s, and 70 ℃ for 60 s. The 807-bp PCR product was genotyped according to their sequences.

Statistical analysis

The software SPSS 17.0 was used for statistical analyses in this study. Descriptive statistics were applied to describe patients' baseline characteristics. The correlation between *T393C* genotypes and the clinical outcome was evaluated by Kaplan-Meier plots and the log-rank test. The survival time was calculated from the date of the primary diagnosis to the end of follow-up or date of death, whichever occurred first. The independent influence of *T393C* SNP and other covariates on survival rates was assessed in multivariate analysis using the Cox regression hazard model. *P* values < 0.05 were considered statistically significant. The compatibility with the Hardy-Weinberg equilibrium was calculated with HWE program (http://linkage.rockefeller.edu/ott/linkutil.htm).

RESULTS

Analysis of GNAS1 T393C genotypes and associated clinicopathological features

The clinicopathological characteristics of patients with genotype distribution are shown in Table 1. There were 94 advanced NSCLC patients participating in this study, including 23 women and 71 men. The average age of participants was 58.6 years (range, 31 to 80 years).

Among 94 patients, 38 (40%) displayed a TT genotype, 29 (31%) with a CT genotype and 27 (29%) with a CC genotype. In the entire patient group, the frequency of the C allele (fC) was 0.55. The distribution was compatible with the Hardy-Weinberg equilibrium. There was no significant correlation between the *GNAS1 T393C* genotypes and clinicopathological parameters, such as age ($P = 0.48$), gender ($P = 0.42$), PS ($P = 0.30$), smoking status ($P = 0.44$) or pathology ($P = 0.59$) (Table 2). Further analysis showed that there was no significant correlation of overall survival (OS) with age ($P = 0.135$), gender ($P = 0.0580$), PS ($P = 0.658$), smoking ($P = 0.473$), pathology ($P = 0.559$), or treatment mode ($P = 0.116$).

Table 1 Clinicopathological characteristics of 94 patients with non-small cell lung cancer

	Subgroup	*n*	MST	1-yr OS (%)	2-yr OS (%)	*P*
Gender	Male	71	14	59	36	0.058
	Female	23	13	53	28	
Age	⩾ 60 yr	51	13	61	26	0.135
	< 60 yr	43	16	52	36	
PS	⩾ 2	25	13	51	25	0.658
	< 2	69	17	64	32	
Smoking	Yes	23	13	55	29	0.473
	No	71	14	58	32	
Pathology	Adenocarcinoma	48	13	54	36	0.559
	Squamous cell carcinoma	46	14	63	29	
Treatment	Supportive treatment only	12	10	49	25	0.116
	Chemotherapy	14	13	56	32	
	Radiotherapy	11	13	60	30	
	Chemoradiotherapy	57	16	64	35	
GNAS1 T393C	TT	38	25	76	48	0.01
	TC	29	12	54	18	
	CC	27	8	23	15	
	TC + CC	56	11.5	25	16	0.01

OS: Overall survival; PS: Performance status.

Table 2 Association between *GNAS1 T393C* single nucleotide polymorphism and clinical parameters

	Subgroup	All (*n* = 94)	TT (*n* = 38; 40%)	TC (*n* = 29; 31%)	CC (*n* = 27; 29%)	*P*
Gender	Male	71	31 (43.6)	22 (30.9)	18 (25.5)	0.42
	Female	23	7 (30.4)	7 (30.4)	9 (39.2)	
Age	⩾ 60 yr	51	22 (43.1)	13 (25.5)	16 (31.4)	0.48
	< 60 yr	43	16 (37.2)	16 (37.2)	11 (25.6)	
Performance status	⩾ 2	25	13 (52.0)	5 (20.0)	7 (28.0)	0.30
	< 2	69	25 (36.2)	24 (34.8)	20 (29.0)	
Smoking	Yes	23	12 (52.2)	6 (26.1)	5 (21.7)	0.44
	No	71	26 (36.6)	23 (32.4)	22 (31.0)	
Pathology	Adenocarcinoma	48	19 (39.6)	17 (35.4)	12 (25.0)	0.59
	Squamous cell carcinoma	46	19 (41.3)	12 (26.1)	15 (32.6)	

GNAS1 T393C TT genotype predicts favorable survival

The median survival of carriers of TT, CT and CC genotypes was 25, 12, and 8 mo, respectively. We analyzed the relationship between overall survival rate and *T393C* genotypes using Kaplan-Meier survival curves. Our data showed that the favorable *TT* genotype was significantly associated with better OS (2-year OS: 48%; $P = 0.01$) when compared with the other genotypes. For example, the 2-year OS for CT genotype was 18% and 15% for CC genotype (Figure 1). By applying the multivariate Cox proportional hazards model, we found that *GNAS1 T393C* polymorphism was independently associated with OS after adjusting the clinicopathological factors ($P < 0.05$). However, the dichotomization between C-genotypes (CC + CT) and T-genotypes (TT) indicated significant lower survival rates for C-allele carriers ($P = 0.01$), which had a 2-year OS rate of 16% (Figure 2).

DISCUSSION

Lung cancer is the major cause of cancer death in the world and there is an urgent need to accurately and individually treat patients with lung cancer. Although clinicopathological parameters such as UICC stage may serve as prognostic markers in lung cancer, it is still desirable to develop more reliable and accurate biomarkers to more precisely predict the clinical outcome of individual patients. Most prognostic biomarkers are developed according to the features of the tumor tissue itself. The *GNAS1* gene encodes the Gαs subunit of G protein and it has been shown that the *GNAS1 T393C* polymorphism correlates with lung cancer[20]. Hence, we investigated whether *GNAS1 T393C* polymorphism can be used to predict the clinical outcome in patients with NSCLC. Our study clearly indicated that the homozygous TT genotype patients had a much higher survival rate than patients with either homozygous CC or heterozygous CT genotype. If we could identify patients with poor clinical outcome, we might develop novel treatment strategies accordingly at the initial stage of management, which could lead to improved individual therapy strategies with higher survival rates. Meanwhile, our results also indicated the potential role of the *GNAS1 T393C* polymorphism as a possible general genetic marker for tumor progression and survival since T-allele carriers demonstrated better clinical outcome than C-allele carriers (TC and CC genotypes). However, it should be noted that the connection between *GNAS1 T393C* polymorphism and survival was

Table 3 The effect of *GNAS1 T393C* on distinct carcinomas

Author	Year	Cancer type	All	Genotype	*n*	OS (%)	Benefit	*P*
Alakus	2009	Gastric cancer	122	TT	26	56.9	TT	0.043
				TC	57	32.7		
				CC	39	42.6		
Schmitz	2007	Cholangiocarcinoma	87	TT	15	10	C+	0.04
				TC	41	17		
				CC	31	18		
Lehnerdt	2008	Laryngocarcinoma	157	TT	40	76	TT	0.037
				TC	75	49		
				CC	42	43.5		
Frey	2006	Chronic lymphocytic leukemia	144	TT	27	73	T+	0.013
				TC	72	63.3		
				CC	45	33.2		
Vashist	2011	Esophageal cancer	190	TT	38	19	CC	0.001
				TC	96	15		
				CC	56	51		
Frey	2005	Bladder cancer	254	TT	49	82	TT	0.015
				TC	121	60		
				CC	84	58		
Frey	2006	Renal cancer	150	TT	34	91	TT	0.01
				TC	79	81		
				CC	37	69		
Frey	2005	Colorectal cancer	151	TT	36	87.8	TT	0.009
				TC	72	71		
				CC	43	50		
Otterbach	2007	Breast cancer	279	TT	64	23	CC	0.01
				TC	162	40		
				CC	53	63		
Lehnerdt	2008	Oral carcinoma	202	TT	48	51.3	TT	0.015
				TC	89	44.7		
				CC	65	36.8		
Kaderi	2008	Chronic lymphocytic leukemia	279	TT	80	65	NS	0.802
				TC	115	70		
				CC	84	64		
Frey	2010	Malignant melanoma	328	TT	69	87.1	TT	0.017
				TC	149	NS		
				CC	110	66		
Xie	2013	Non-small cell lung cancer	131	TT	33	NS	TT	0.02
				TC	63	NS		
				CC	35	NS		

OS: Overall survival.

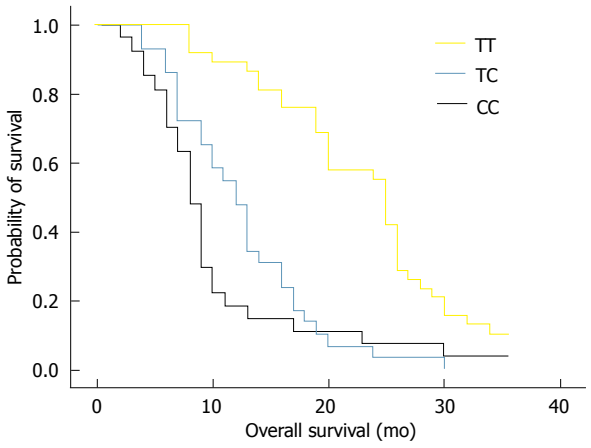

Figure 1 The overall survival of 94 lung cancer patients according to *GNAS1 T393C* genotypes. The data were analyzed by Kaplan-Meier analysis, $P < 0.01$, TT genotype *vs* other genotypes.

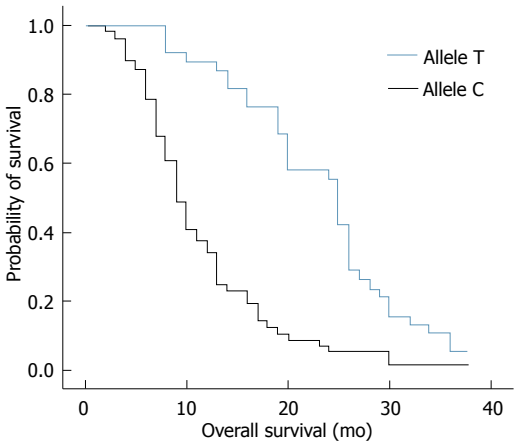

Figure 2 The overall survival of 94 lung cancer patients according to *GNAS1 T393C* genotype with dichotomization between C+ and C- genotypes, $P < 0.01$.

different in different types of tumors. For some tumors, TT genotype was significantly correlated with better OS compared with CT or CC genotype. For example, in advanced squamous cell carcinoma of the larynx, the five-

year survival rate for TT genotype patients was 76%, 49% for TC genotype, and 43.5% for CC genotype[10]. Also, it had been reported that the five-year survival rate of sporadic colorectal cancer patients with a TT genotype (87.8%) was much higher than that of patients with a TC (71.0%) or CC genotype (50.0%)[15]. On the other hand, in intrahepatic cholangio-carcinoma[9], esophageal cancer[12] and breast cancer[16], the patients with a CC genotype had a more favorable clinical outcome (Table 3). Thus, it was conceivable that the *GNAS1 T393C* polymorphism in various tumor types had different biological effects. In order to understand the significance of the T393C genotypes in different tumor types, further more studies are needed to clarify the molecular mechanisms.

In vitro studies demonstrated that increased Gαs expression promotes apoptosis[21]. Therefore, it is highly likely that increased Gαs expression and the subsequently increased apoptosis could be associated with better survival rate in patients with a GANS1 *TT* genotype. *In vitro* experiments also suggest that the product of Gαs, cyclic AMP, could play a crucial role in the proapoptotic process. It has been reported that increasing the intracellular concentration of cyclic AMP leads to enhanced apoptosis in several cell lines including lymphoma cells[5], leukemic[22] and ovarian cancer cells[23]. Gαs was also found to be differentially expressed between various *GNAS1 T393C* genotypes. Previous studies have suggested that Gαs transcription level is increased in individuals with a *GNAS1 393 TT* genotype[13]. Intriguingly, the mRNA stability has been shown to be determined by the coding region of some genes[24-26]. Using the MFOLD (the software for the prediction of the secondary structure of single stranded nucleic acids), Alakus *et al*[8] have reported that the substitution of T393 to C affects the structure of mRNA, most likely the mRNA folding.

Several biomarkers have been used as predictive and prognostic markers for NSCLC patients. A prognostic biomarker is a molecule that can be used to indicate the patient survival independent of the treatment received. In other words, it is an indicator of the innate tumor aggressiveness. For example, KRAS mutations can serve as a good prognostic biomarker indicating the poor survival for NSCLC patients when compared with the patients without KRAS mutations, independent of therapy. Xie *et al*[20] has reported that the *GNAS1 T393C* polymorphism can somehow predict the chemotherapy sensitivity and overall survival rate in advanced NSCLC patients treated with gemcitabine and platinum[20]. Here, our data clearly showed that the *GNAS1 T393C* TT genotype was prognostic of better overall survival for NSCLC patients, independent of therapy.

Nevertheless, it should be emphasized that in this study, we only investigated a small population of patients. Although our study indicated that genetic host factors play a role in tumor progression, which was consistent with the previously published data[20], further independent studies of large cohorts are necessary to confirm the reliability of our findings. Furthermore, the molecular mechanisms underlying the significance of the *GNAS1 T393C* genotype associated with potentially surrogate SNPs remain to be explored.

COMMENTS

Background

Lung cancer is major cause of cancer death around the world. Although some clinicopathological parameters like UICC stage may be used as prognostic biomarkers in lung cancer, other reliable markers that can help precisely predict the clinical outcome of individual patients are still desirable. Most prognostic biomarkers are based on features of the tumor tissue itself.

Research frontiers

Characterization of single nucleotide polymorphisms (SNPs) as a prognostic biomarker in cancer has become the hotspot of recent research. The *T393C* polymorphism of the *GNAS1* gene is one such polymorphism.

Innovations and breakthroughs

Several molecular markers have been used as predictive and prognostic markers for non-small cell lung cancer (NSCLC). A prognostic biomarker is a biomolecule that can be used to indicate the patient survival independent of the treatment received. It can also indicate for the innate tumor aggressiveness. For example, the KRAS mutations are prognostic of poor survival for NSCLC patients when compared to the absence of KRAS mutations, independent of therapy. Xie *et al* reported that the *GNAS1 T393C* polymorphism can be used to predict the chemotherapy sensitivity as well as the survival rates in advanced NSCLC patients treated with gemcitabine and platinum. Here, the data clearly indicate that the *GNAS1 T393C* TT genotype was prognostic of better survival rates for NSCLC patients, independent of therapy.

Applications

The identification of patients with high-risk lung cancer could help develop novel and individual treatment strategies and could improve the clinical outcome. This data clearly indicate that genetic polymorphism in the *GNAS1 T393C* influenced survival in advanced non-small lung cancer with a worse clinical outcome for C allele patients.

Terminology

SNPs refer to a DNA sequence variation occurring commonly within a population (*e.g.*, 1%) in which a single nucleotide -A, T, C or G - in the genome (or other shared sequence) differs between members of a biological species or paired chromosomes.

Peer review

The manuscript is comprehensive and important.

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CASE REPORT

Carcinomatous meningitis due to gastric adenocarcinoma: A rare presentation of relapse

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Abstract

While solid tumors are less commonly associated with meningeal involvement; lung, breast and melanoma are the ones most often reported. A few case reports have included gastric carcinoma but these are rare and most often associated with systemic disease at the time of diagnosis. Here we report a unique presentation of gastric carcinoma relapse with leptomeningeal carcinomatosis. An 81-year-old female was diagnosed with gastric cancer approximately one year before presentation. Following neoadjuvant chemotherapy, she had gastrectomy. Her periodic surveillance was stable. Thereafter she presented with a one week history of progressive fatigue lightheadedness, syncope. During hospitalization her mental status deteriorated. A repeat computed axial tomography scan of the head showed no changes to suggest an etiology. A lumbar puncture was performed and cerebral spinal fluid (CSF) cytopathology confirmed gastric signet cell adenocarcinoma. Encephalopathy was likely caused by increased intracranial pressure from communicating hydrocephalus. Leptomeningeal carcinomatosis is associated with short life expectancy. Therapeutic lumbar punctures and best supportive care or systemic therapy can be applied with guarded prognosis. Survival, however, may improve with cytologic negative conversion of the CSF if patient performance status allows treatment.

Key words: Gastric cancer; Gastric adenocarcinoma; Leptomenigeal carcinomatosis; Signet cell adenocarcinoma; Carcinomatous meningitis

Core tip: Solid tumors rarely have leptomeningeal carcinomatosis. Gastric adenocarcinoma have been rarely reported with leptomeningeal involvement, and most of the reports have been documented in patients with Asian heritage, who have higher incidence of gastric adenocarcinoma. This leptomeningeal involvement is usually a late complication of disseminated and relapsed disease. In this case, however, we describe a patient with recurrence of gastric adenocarcinoma that presented with leptomenigeal carcinomatosis. We describe the presentation, the pertinent medical history of the patient, and the clinical outcome of the disease. We highlighted that leptomeningeal carcinomatosis can be a presentation of relapse, which is rare, and indicates a poor prognosis.

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INTRODUCTION

Leptomeningeal carcinomatosis (LMC) is defined as the infiltration of the pia mater and the arachnoid membrane

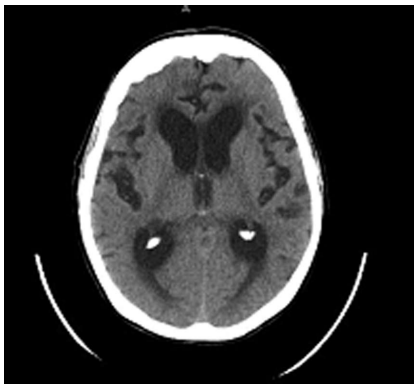

Figure 1 Computed tomography scan of the head at time of admission shows prominence of the ventricular system, slightly out of proportion to the sulcal prominence.

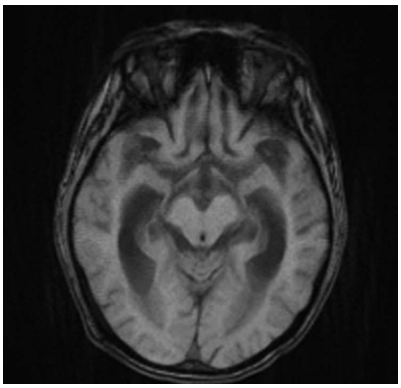

Figure 2 Magnetic resonance imaging of the brain with no evidence of leptomeningeal enhancement.

by malignant cells. LMC can be limited to the meninges but can also occur in association with paranchymal invasion of the central nervous system (CNS) with or without dissemination into the ventricles. LMC is an underestimated complication of malignancy. It is estimated to occur in 5%-8% of cancer patients[1]. However, the clinical diagnosis of LMC has been identified in 2%-4% of the patients who found to have LMD on autopsy[2].

The incidence of LMC varies by the primary site of malignancy. LMC occurs in 78% of hematologic malignancies if prophylactic treatment is not administered. It is less common in solid tumors, of which lung cancer (9%-25%), breast cancer (2%-5%), and melanoma (up to 23%) are the most common[3,4].

Gastric cancer complicated by LMC is very rare. It is estimated to occur in 0.16% of all cases of gastric cancer[5], of which 87% have disseminated disease[6]. We present a case of recurrence of gastric cancer manifested by LMC on presentation.

CASE REPORT

An 81-year-old woman was diagnosed with signet cell adenocarcinoma. Staging Positron emission tomography/computed tomography (CT) scan showed increased fluoro-D-glucose activity in the stomach and regional lymph nodes without distant metastasis. She was treated with neoadjuvant chemotherapy using Epirubicin, Oxaliplatin and Capecitabine for 6 cycles. This was followed by total gastrectomy with Roux-en-Y esophagojejunostomy. Pathologic staging showed pT2, N0 with negative margins and 23 negative lymph nodes. She received no adjuvant therapy, and active surveillance was initiated.

One year later, she presented with a one week history of progressive lightheadedness, rigors, generalized fatigue and an episode of syncope. Her review of systems was notable for drenching night sweats for about 3 mo.

On examination, she was afebrile, weak, alert and oriented to herself. Examination of the cranial nerves was normal, sensory and motor examination were normal without ataxia or nuchal rigidity.

CT scan of the brain showed low attenuation changes of the left cerebellar hemisphere, likely sequela of remote vascular insult. There was prominence of the ventricular system, slightly out of proportion to the sulcal prominence which raised the possibility of normal pressure hydrocephalus (Figure 1).

Ten days prior to admission, a surveillance CT scan of the abdomen and the pelvis showed small amount of fluid within the abdomen and the pelvis, and nodules on the gastrectomy bed and the omentum which were stable compared with previous CT scan.

During the course of her hospital stay the patient was found to have pyuria and atrial fibrillation with rapid ventricular response. Rate control was instituted and she received empiric antibiotic therapy for urinary tract infection. Her blood cultures were negative and her urine cultures grew enterococci. While on therapy, she had progressive deterioration of her mental status and became non-verbal and unarousable. An enhanced magnetic resonance imaging of the brain with contrast showed no evidence of leptomeningeal enhancement (Figure 2). Electroencephalogram, vitamin B12, thyroxin stimulation hormone, folic acid and ammonia level were unrevealing. A diagnostic lumber puncture was performed revealing an opening pressure of 38 cm water. Cerebrospinal fluid (CSF) analysis showed WBC of 31 with 55% lymphocytes, 1% polymorphonuclear white cells, 36% atypical cells, protein of 120 mg/dL, and glucose of 85 mg/dL. Cytologic examination confirmed CSF involvement with adenocarcinoma with signet cell features (Figures 3 and 4). CSF microbiologic workup was negative. Best supportive care and therapeutic lumber punctures were applied to relieve the communicating hydrocephalus. The patient, however, continued to deteriorate and died shortly thereafter. Autopsy was refused by the family and there decision was respected.

DISCUSSION

In our case, we present a patient with signet cell gastric adenocarcinoma. This histologic subtype is the most among solid tumors to be complicated with LMC[7]. Among patients diagnosed with LMC in solid tumors, the most common encountered solid tumors are breast, lung,

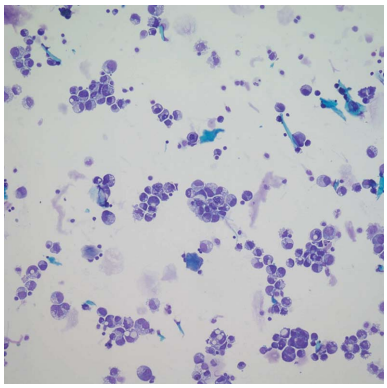

Figure 3 Signet ring adenocarcinoma cells in the cerebral spinal fluid.

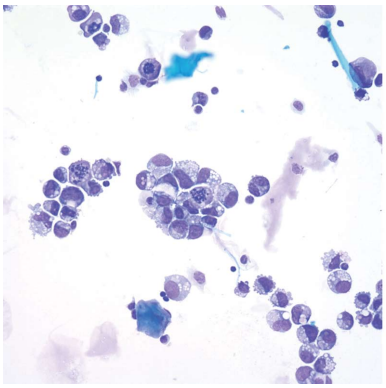

Figure 4 Signet ring adenocarcinoma cells in the cerebral spinal fluid.

and melanoma[8]. However, In east Asian countries LMC due to gastric carcinoma is more common compared to other places due to higher incidence of this malignancy in that region[9]. Many cases of LMC have been reported in the contest of gastric cancer in advanced stage and poorly differentiated or signet-ring cell histology[10]. Although our patient did not have clinical or histological systemic dissemination at the time of leptomeningeal relapse, she had increased intraperitoneal fluid and nodularity at the gastrectomy bed and the omentum, which may reflect local recurrent disease which was interpreted as stable and left for monitoring. Our case is unique in that the patient presented with altered mental status secondary to LMC as the principle and first manifestation of Signet Cell Gastric Adenocarcinoma recurrence.

The increase in survival of patients with signet cell gastric adenocarcinoma with the discovery of newer therapeutic agents, and the poor penetration of these drugs into the CNS has led to an increase in the number of cases of LMC associated with gastric adenocarcinoma[11-13]. Prognosis of LMC is guarded, particularly in solid tumors. Treatment options are limited, including intrathecal therapy, systemic therapy and best supportive care.

Overall survival in cases of LMC is very short with median overall survival of 4-5 wk without treatment and 2-4 mo with treatment[7,14,15]. Our patient survived less than 2 wk from the time of diagnosis. One study noted that clearance of the CSF with intrathecal (IT) chemotherapy is independently associated with longer survival[6].

Radiotherapy has been tried for treatment; its role, however, remains controversial[16]. Systemic chemotherapy usually is given after IT chemotherapy in patients with good performance status. Due to the poor performance status of our patient, best supportive care was deemed appropriate.

We conclude that, despite LMC being a rare manifestation of recurrent gastric cancer, it should be considered in the differential diagnosis of a patient presenting with encephalopathy with prior history of gastric cancer. LMC may be the first sign of relapse in stomach cancer. LMC in the context of gastric cancer has poor prognosis[2]. Therapeutic options should be individualized based on the performance status of the affected patient. Further studies are needed to determine the risk factors for LMC in the contest of gastric cancer and to formulate an early diagnosis and more effective treatment.

COMMENTS

Case characteristics

An 81-year-old female with a history of gastric adenocarcinoma in remission, presented with a one week history of progressive fatigue, lightheadedness and syncope.

Clinical diagnosis

Encephalopathy.

Differential diagnosis

Meningitis, encephalitis, metabolic encephalopathy, hydrocephalus.

Laboratory diagnosis

Complete blood count, comprehensive metabolic panel, vitamin B12, thyroxin stimulation hormone, folic acid and ammonia level were normal.

Imaging diagnosis

Computed tomography scan of the brain showed prominence of the ventricular system, slightly out of proportion to the sulcal prominence, which raised the possibility of normal pressure hydrocephalus while enhanced magnetic resonance imaging of the brain with contrast showed no evidence of leptomeningeal enhancement.

Pathological diagnosis

Cerebral spinal fluid analysis showed atypical cells which were identified as adenocarcinomatous cells with signet cell features.

Treatment

Best supportive care and therapeutic lumber punctures to relieve the communicating hydrocephalus were applied.

Related reports

There are many case reports of leptomeningeal carcinomatosis in the contest of gastric adenomarcima, However this case was unique because the relapse of adenocarcinoma was manifested by acute encephalopathy.

Term explanation

Leptomeningeal carcinomatosis (LMC) is infiltration of the pia mater and the arachnoid membrane by malignant cells

Experiences and lessons

LMC can be the first sign of relapse of gastric adenocarcinoma. LMC in the context of gastric cancer has poor prognosis

Peer review

This is an interesting case report.

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