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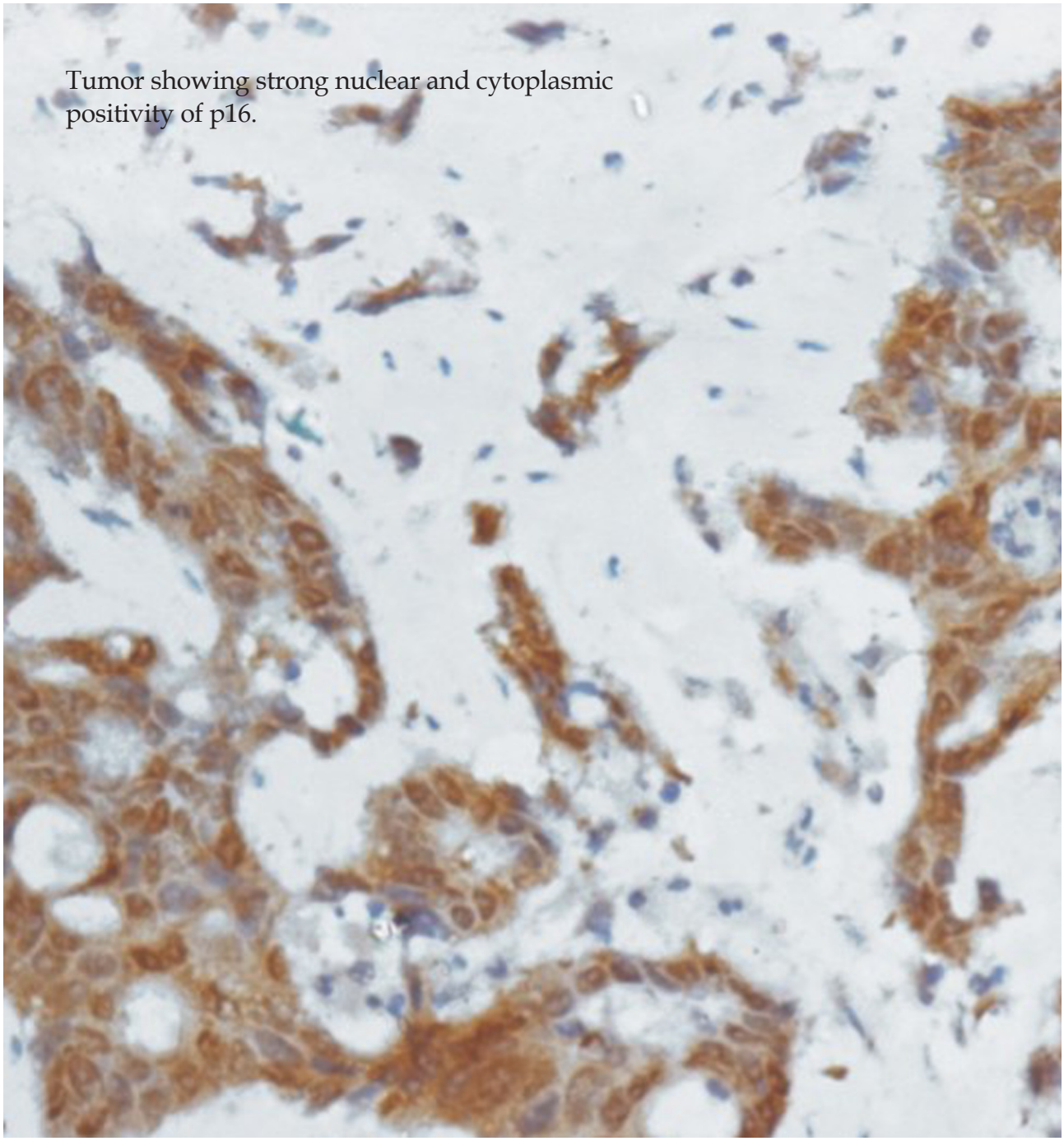

Tumor showing strong nuclear and cytoplasmic positivity of p16.

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EDITORIAL

Recent advances in chemotherapy for advanced gastric cancer

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Abstract

Although medical treatment has been shown to improve quality of life and prolong survival, no significant progress has been made in the treatment of advanced gastric cancer (AGC) within the last two decades. Thus, the choice of optimum standard first-line chemotherapy regimen for AGC remains debatable, and most responses to chemotherapy are partial and of short duration, with a median survival of approximately 7-11 mo and survival at 2 years rarely more than 10%. Recently, remarkable progress in tumor biology has led to the development of new agents that target critical aspects of oncogenic pathways. For AGC, several molecular targeting agents are now under evaluation in international randomized studies, and trastuzumab, an anti-HER2 monoclonal antibody, has shown antitumor activity against HER-2 positive AGC. However, this benefit is limited to only about 20% of patients with AGC (patients with HER-2 positive AGC). Therefore, there remains a critical need for both the development of more effective agents and the identification of predictive and prognostic molecular markers to select those patients who will benefit most from specific chemotherapeutic regimens and targeted therapies.

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Key words: Gastric cancer; Prognosis; Chemotherapy; Cytotoxic agents; Targeted agents

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INTRODUCTION

The survival of patients with gastric cancer is substantially worse than that of patients with most other solid malignancies, and the only treatment that offers a potential cure is complete resection of the tumor. However, since the disease is asymptomatic in its early stages, more than half of gastric carcinomas are diagnosed in the advanced stage, when resection is no longer possible. Thus, although medical treatment has been shown to improve quality of life and prolong survival, there has been no significant progress in the treatment of advanced gastric cancer (AGC) within the last two decades[1,2]. The choice of optimum standard first-line chemotherapy regimen for AGC remains debatable, and most responses to chemotherapy are partial and of short duration. As a result, the current median survival is approximately 7-11 mo and survival at 2 years is rarely more than 10%[3].

These facts notwithstanding, an emerging understanding of the molecular pathways that characterize cell growth, cell cycle, apoptosis, angiogenesis, and invasion has provided novel targets in cancer therapy, leading to the development of therapeutic strategies including epidermal growth factor receptor (EGFR) inhibitors, anti-angiogenic agents, cell cycle inhibitors, and apoptosis promoters. For AGC, several molecular targeting agents are now under evaluation in international randomized studies, and trastuzumab, an anti-HER2 monoclonal antibody, has been shown to exhibit antitumor activity against HER-2 positive AGC. Accordingly, this review covers the recent advances, including biologic agents, in the first-line treatment of AGC on the basis of the best available evidence.

OVERVIEW OF "CLASSICAL" CHEMOTHERAPY

At least two phase Ⅲ randomized clinical trials and one meta-analysis have shown that in AGC, systemic chemotherapy leads to improvement in survival and symptoms when compared with best supportive care alone[1-3]. The question of treating AGC with a single agent *vs* combination chemotherapy has been addressed by randomized studies with a total of 1472 patients, pooled in Wagner's meta-analysis[1,4-7]. Most of the studies used 5-fluorouracil (5-FU) in the single-agent arm. The resulting HR of 0.83 (95% CI: 0.74-0.93) for survival in favor of combination chemotherapy provides evidence of a statistically significant survival benefit with combination *vs* single-agent chemotherapy. Although the overall treatment-associated toxicities were higher in the combination chemotherapy arms, this was usually not statistically significant in the individual trials.

Whilst there is no international agreement accepting any particular schedule as the standard of care for AGC, there is a body of evidence coming from randomized trials and one meta-analysis that should be underlined. In several Korean and Japanese randomized trials comparing 5-FU alone with 5-FU based combination regimens, the response rates and progression-free survival in the cisplatin + 5-FU (CF) arm were better than those for the single-agent 5-FU although no combination regimen demonstrated survival prolongation[6, 8-10](Table 1). However, the interpretation of these results, particularly for determining the reference arms of subsequent studies, differed among regions. In most countries other than Europe and Japan, CF was regarded as the reference arm, as the activity of this monotherapy was limited, with a response rate of around 10% and median progression-free survival of around 2 mo. Meanwhile, triplet regimens have been commonly used in Europe. A significant increase in survival (median 6.1 mo *vs* 8.7 mo, $P = 0.0005$) was observed in a trial by Webb *et al*[11] who compared ECF (epirubicin + CF) *vs* FAMTX (5-FU adriamycin + methotrexate). In another phase III trial that compared ECF and Mitomycin-CF, ECF was superior in terms of quality of life and showed similar results to those of Webb's trial for both the overall response rate (42%) and survival (median 9.4 mo)[12]. Thus, given these results, ECF is currently considered by many oncologists in Europe as the standard treatment. Despite a recent meta-analysis with a subanalysis including 501 patients (treated with CF or CF plus an anthracycline) which showed a significant improvement in overall survival when an anthracycline was added to CF (HR: 0.77, 95% CI: 0.62-0.95)[3], both CF and ECF can still only be considered as reference regimens, as there have been no phase Ⅲ studies comparing them directly. Although these regimens have been found to obtain responses in 20%-40% of patients, the response duration is short, with very few complete responses (approximately 5%), the median time to progression (TTP) is about 4-5 mo and the median survival does not exceed 7-10 mo.

Table 1 Treatment results of cisplatin/5-fluorouracil for advanced gastric cancer in randomized trials

	Korea[8]	Japan[6]	EU[9]	US/EU[10]
No. of patients	105	103	134	112
Response rate (%)	34	51	20	23
Median progression-free survival (mo)	3.9	5.0	4.1	3.7
Median overall survival (mo)	7.3	8.5	7.2	8.5

EU: European Union; US: United States.

Prognostic factors

Prognostic factors are important when designing and interpreting therapeutic trials related to human tumors. In general, it is accepted that for advanced gastric or gastroesophageal cancer neither the primary tumor location nor the histological type has any prognostic impact on survival. In a series of 1080 patients with gastric or gastroesophageal cancer treated in three consecutive trials between 1992 and 2001, the probability of responding to chemotherapy was significantly reduced for individuals with a performance status (PS) of 2, liver or peritoneal metastases, and high serum levels of alkaline phosphatase[13]. Meanwhile, in other series of Korean patients with metastatic gastric cancer, the importance of a poor PS and elevated alkaline phosphatase as negative predictors has been confirmed[14,15]. In addition, a multivariate analysis underlined the significance of other negative findings, including the presence of ascites, serum albumin < 3.6 g/dL, bone metastasis, and the absence of primary tumor resection. Thus, the stratification of patients in randomized trials according to well-established prognostic factors can avoid bias and may allow a better balance between different study arms.

CHEMOTHERAPY WITH NEW CYTOTOXIC AGENTS

Given the limitations of classical chemotherapy combinations in this setting, recent studies have focused on examining the role of chemotherapy with new cytotoxic agents, in particular, docetaxel, irinotecan, oxaliplatin, paclitaxel, and oral fluoropyrimidines (capecitabine and S-1) (Table 2).

Table 2 Results of recent randomized trials with new cytotoxic agents

Study	Treatment	*n*	RR (%)	TTP (median, mo)	MST (mo)	*P* value
Van Cutsem *et al* (V325)[10]	CDDP + 5-FU	224	25	3.7	8.6	0.02
	D + CDDP + 5-FU	221	37	5.6	9.2	
Dank *et al* (V306)[16]	CDDP + 5-FU	163	26	4.2	8.7	NS
	I + 5-FU/LV	170	32	5.0	9.0	
Kang *et al* (ML17302)[17]	CDDP + 5-FU	137	29	5.0	9.3	NS
	CDDP + X	139	41	5.6	10.5	
Cunningham *et al* (REAL-2)[18]	ECF	263	41	6.2	9.9	NS
	EOF	245	42	6.5	9.3	
	ECX	250	46	6.7	9.9	
	EOX	244	48	7.0	11.2	
Boku *et al* (JCOG9912)[19]	5-FU	234	9	2.9	10.8	NS
	CDDP + I	236	38	4.8	12.3	
	S-1	234	28	4.2	11.4	
Koizumi *et al* (SPIRITS)[20]	S-1	150	31	4.0	11.0	NS
	CDDP + S-1	148	54	6.0	13.0	

C: Cisplatin; D: Docetaxel; E: Epirubicin; F: 5-fluorouracil; I: Irinotecan; O: Oxaliplatin; X: Capecitabine; MST: Median survival time; TTP: Time to progression.

Docetaxel

Since several phase Ⅱ studies have shown that docetaxel, alone or in combination with cisplatin, is active against AGC, the addition of docetaxel to a doublet including cisplatin and 5-FU (DCF) was studied in a single international randomized trial (V-325).The results of this phase Ⅲ trial indicated a better response rate, longer progression-free survival (median, 5.6 mo *vs* 3.7 mo; $P = 0.0004$), and significantly prolonged overall survival (median, 9.2 mo *vs* 8.6 mo; $P = 0.0201$) for those patients receiving the DCF triplet[10]. DCF caused a higher levels of toxicity symptoms, including neutropenia (grade 3-4, 82% *vs* 57%), febrile neutropenia (29% *vs* 12%), and diarrhea (grade 3-4, 19% *vs* 8%). However, no significant differences were observed in treatment-related death. Although the quantitative benefit for survival was limited, this trial did show for the first time in AGC that DCF can improve the quality of life parameters and induce a more tangible clinical benefit over the control arm[21,22]. In addition, DCF significantly prolonged the time to definitive worsening of Karnofsky PS when compared with CF. Therefore, these findings indicate that DCF can also be considered as a therapeutic option for patients with AGC who have a PS of 0-1 and can tolerate this drug combination. Furthermore, different combinations with capecitabine, S-1, and irinotecan have also been examined in phase Ⅱ studies, with interesting results[23-26].

Irinotecan

The two most tested combinations for AGC have been irinotecan with cisplatin and irinotecan in combination with either leucovorin and 5-FU bolus or as a continuous infusion: ILF/FOLFIRI-either the AIO regimen (ILF) or the DeGramont regimen (FOLFIRI). The most important study is V-306[16], although the results of two randomized phase Ⅱ studies reported by Bouche and Moehler are also interesting[27,28]. Similar to V-325, the V-306 study was designed in two phases, beginning with a randomized phase Ⅱ trial that was then used to select the experimental arm in the subsequent phase Ⅲ trial. The international phase Ⅲ study comparing ILF with CF demonstrated a trend toward a longer TTP and superior overall survival with the ILF regimen, although the differences were not statistically significant (HR, 1.23 and 1.08, respectively). The median TTP for the ILF and CF arm was 5.0 and 4.2 mo, respectively, and the median overall survival was less than 10 mo in both arms. Therefore, the authors concluded that ILF without cisplatin could be considered as a reasonable alternative first-line treatment option, although it provided no definite advantage in efficacy over CF.

Oxaliplatin

A variety of different oxaliplatin combinations have been studied, and all have been associated with response rates in the range of 40%-67%, with median survival durations between 9 and 15 mo[29-31]. At least two trials have directly compared oxaliplatin-based *vs* cisplatin-containing regimens (including ECF), resulting in a comparable efficacy, yet different toxicity profiles.

The substitution of oxaliplatin for cisplatin in combination with epirubicin and a fluoropyrimidine was investigated in the REAL-2 trial, a randomized phase Ⅲ comparison of ECF, ECX, EOF, and EOX[18]. In the final report, the response rates in the two oxaliplatin-containing arms were comparable to those achieved with the two cisplatin-based regimens, and no significant differences were noted in the median survival. However, when the four groups were considered separately, the median survival for the patients treated with EOX was modestly longer than that with ECF (median 11.2 mo *vs* 9.9 mo, HR = 0.80). Furthermore, the patients in both oxaliplatin-containing arms had significantly less grade 3 to 4 neutropenia, alopecia, thrombocytopenia, and renal dysfunction, although they had more peripheral neuropathy and diarrhea.

Similar outcomes were found when substituting oxaliplatin for cisplatin in a randomized phase Ⅲ trial comparing the FLO regimen (infusional 5-FU, leucovorin, and oxaliplatin) and FLP regimen (5-FU, leucovorin, and cisp-

latin)[32]. No statistically significant differences were noted between the two arms in terms of the response rates of 34% and 25%, respectively, or TTP (primary end point) of 5.7 and 3.8 mo, respectively. From a toxicity standpoint, FLO was associated with less nausea and vomiting, fatigue, renal toxicity, and alopecia, yet more grade 3 or 4 sensory neuropathy. Thus, when taken together, these data show that oxaliplatin combinations are at least as effective as cisplatin, and have a more favorable toxicity profile than cisplatin.

Capecitabine

Since both CF and ECF regimens require central venous access and an ambulatory infusion pump, orally active fluoropyrimidines, including capecitabine and S-1, have been actively studied to improve the convenience of combination regimens. A randomized, non-inferiority trial comparing 21 d of capecitabine (1000 mg/m^2 twice daily for 14 d) plus cisplatin (80 mg/m^2 on day 1) with infusional 5-FU (800 mg/m^2 per day, days 1-5) plus the same dose of cisplatin, demonstrated that capecitabine was not inferior to 5-FU: the median TTP and median overall survival were 5.6 and 10.5 mo in the capecitabine/cisplatin arm and 5.0 and 9.3 mo in the CF arm, respectively[17]. Similar results were observed in the REAL-2 trial[18], a randomized phase Ⅲ study comparing capecitabine plus fluorouracil with oxaliplatin plus cisplatin. No significant differences were noted among the groups in terms of the objective response rate, although a statistically non-significant trend towards improved overall survival was found when the outcomes of both capecitabine-containing arms were combined and compared to both 5-FU-containing arms (HR for death 0.86, 95% CI: 0.8-0.99). However, the toxicity profile with capecitabine was different. The patients receiving ECX (epirubicin, cisplatin, and capecitabine) had a higher rate of grade 3 or 4 neutropenia than patients who received ECF (epirubicin, cisplatin, and 5-FU) (51.5% *vs* 41.7%), while the EOX group had a significantly lower rate (27.6%). However, the rates of febrile neutropenia were not significantly different between the arms. The incidence of grade 3 or 4 hand-foot syndrome was higher with ECX than with ECF or EOX (10.3% *vs* 4.3% *vs* 3.1%, respectively).

Thus, based on these results, the substitution of capecitabine for infusional 5-FU in these regimens results in outcomes which are at least equivalent in terms of efficacy. Moreover, the use of capecitabine allows patients to avoid infusion pumps and a central venous catheter, although the cost of capecitabine is significantly higher than that of 5-FU.

S-1

S-1 is a fourth generation fluoropyrimidine and an oral formulation of tegafur, ftorafur: and 4-dihydroxypyridine: potassium oxonate, in a 1:0.4:1 ratio. Extensive phase Ⅱ/Ⅲ trials of S-1 alone or combination with cisplatin have already been conducted in Japan. In the JCOG 9912 (Japan Clinical Oncology Group) trial comparing 5-FU alone, irinotecan/cisplatin, and S-1 alone, both investigational arms (irinotecan/cisplatin and S-1) showed a significantly higher response rate and longer progression-free survival than the control arm of 5-FU alone[19]. In terms of overall survival, this study demonstrated that S-1 was not inferior to 5-FU monotherapy, with a HR of 0.83. However, there was no demonstration of a significant superiority of irinotecan/cisplatin over 5-FU (HR = 0.85). Meanwhile, in the SPIRITS trial, which compared S-1 monotherapy with a combination of S-1 and cisplatin, the combination arm yielded a significantly higher response rate, and longer progression-free survival and overall survival (HR = 0.774) than the control arm[20]. Thus, on the basis of these Japanese results, S-1 plus cisplatin is the most reasonable standard regimen for AGC in Japan. However, in western countries, the FLAGS trial comparing an experimental regimen of S-1 plus cisplatin (CS arm, S-1: 25 mg/m^2 bid for 21 d followed by a 7-d break; cisplatin: 75 mg/m^2 on day 1, every 4 wk) with a reference regimen of 5-FU plus cisplatin (CF arm, 5-FU: 1000 mg/m^2 as a 5-d continuous infusion; cisplatin: 100 mg/m^2 on day 1, every 4 wk) did not demonstrate a superior overall survival (median overall survival, CS: 8.6 *vs* CF: 7.9), although the CS arm did result in a significantly better safety profile when compared to the CF arm[33]. S-1 displays ethnic differences in its effects on metabolism, leading to differential dose tolerance and toxicity. The tolerable S-1 dose is substantially lower in Western patients than in Asian patients, which may explain its poorer acceptance in Western countries.

CHEMOTHERAPY WITH TARGETED AGENTS

During the past few decades, remarkable progress in tumor biology has led to the development of new agents that target critical aspects of oncogenic pathways. In various tumor types, including hematologic malignancies, colorectal cancer, breast cancer, renal cancer, and gastrointestinal stromal tumor, many molecular targeting agents have already exhibited significant antitumor activity. Therefore, the incorporation of these biologic agents in therapeutic regimens is also being investigated for gastric cancer patients (Table 3).

Epidermal growth factor receptor inhibitors

EGFR family is composed of four members: HER1 (also known as EGFR1), HER2, HER3, and HER4, amongst which EGFR1 and HER2 represent the targets for drugs currently under development for gastric cancer. EGFR is commonly over-expressed in gastrointestinal malignancies, and its over-expression is associated with a more aggressive phenotype and poorer survival, suggesting that EGFR can be a rational therapeutic target[34]. Following poor reports on the efficacy of the tyrosine kinase inhibitors (TKIs) gefitinib and erlotinib in gastric cancers[35,36], monoclonal antibodies, primarily cetuximab, have been tested in several recently published trials[37,38]. In a phase Ⅱ trial (n = 38) using cetuximab in combination with 5-FU, leucovorin, and irinotecan (FOLFIRI) in chemonaive patients with

Table 3 Ongoing phase Ⅲ clinical studies with monoclonal antibodies for gastric cancer

Study	Drug	Indication
ToGA[a][39]	XP or FP ± trastuzumab	Advanced gastric cancer (HER2-positive)
AVAGAST	XP ± bevacizumab	Advanced gastric cancer
REAL-3	EOX ± panitumumab	Advanced esophagogastric cancer
EXPAND	XP ± cetuximab	Advanced esophagogastric cancer
MAGIC-2	Perioperative ECX ± bevacizumab	Operable gastric cancer

[a]Completed trial.

advanced gastric or gastroesophageal junction (GEJ) cancers, an objective response rate of 44% was observed in a population of 89% stomach and 11% GEJ cancers, and the median TTP was 8 mo[38]. Similar to the results with colorectal cancer, the EGFR expression levels did not correlate with the treatment efficacy. Meanwhile, in a biomarker analysis included in the trial by Han *et al*[37], they confirmed that *k-ras* mutations or an increased EGFR gene copy number are uncommon events in gastric cancer. They also demonstrated that patients with EGFR expression and low levels of the major ligands EGF and tumor growth factor-α had a 100% response rate, a finding that deserves urgent confirmation in prospective trials. However, despite a favorable comparison between the reported response rates in these phase Ⅱ trials for combination chemotherapy with cetuximab and current data for chemotherapy alone[18], the median survival is similar to previously published phase Ⅱ clinical trials. Accordingly, an ongoing international phase Ⅲ trial (EXPAND) is expected to define the role of cetuximab in combination with capecitabine and cisplatin in the first-line setting for patients with advanced gastric or GEJ adenocarcinomas.

Trastuzumab is a humanized anti-HER2 monoclonal antibody that is already widely accepted as a standard agent for HER-2 positive breast cancer. In the case of gastric cancer, this agent has also been evaluated in a global randomized trial comparing 5-FU or capecitabine/cisplatin with 5-FU or capecitabine/cisplatin plus trastuzumab, based on the examination of HER-2 overexpression in gastric cancer tissues[39]. Among 3807 patients centrally tested for their HER-2 status, 22.1% were HER-2 positive. The median overall survival was significantly improved in the trastuzumab arm when compared to the chemotherapy alone arm (13.5 mo *vs* 11.1 mo, $P = 0.0048$, HR = 0.74, 95% CI: 0.60-0.91). Plus, the safety profiles were similar with no unexpected adverse events in the trastuzumab arm. Therefore, it was concluded that trastuzumab is a new, effective, and well-tolerated treatment for HER2-positive AGC.

Lapatinib is a dual inhibitor of the tyrosine kinase domains of HER-1 and HER-2, based on its interference with the adenosine triphosphate binding. Lapatinib has also already been shown clinically to be active against HER-2 positive breast cancer, as a monotherapy and in combination with capecitabine. However, a single-agent phase Ⅱ study demonstrated very modest activity with a response rate of only 5% in unselected patients with metastatic gastric cancer[40]. A randomized trial comparing lapatinib and paclitaxel with paclitaxel alone in patients with HER-2 positive metastatic gastric cancer in a second-line setting is ongoing.

Angiogenesis inhibitors

Recognition of the vascular endothelial growth factor (VEGF) pathway as a key regulator of angiogenesis has led to the development of several VEGF-targeting agents, including neutralizing antibodies to VEGF or its receptor (VEGFR), as well as TKIs targeting the VEGFR.

The addition of bevacizumab, a humanized monoclonal antibody against VEGF-A, to chemotherapy has been shown to prolong survival in patients with metastatic colorectal cancer[41]. However, available clinical data on the use of an angiogenesis inhibitor in patients with advanced gastric or GEJ tumors are limited to nonrandomized phase Ⅱ trials using either bevacizumab or sunitinib. Nonetheless, a pivotal phase Ⅱ trial ($n = 47$) using bevacizumab in combination with irinotecan and cisplatin as a first-line therapy in patients with gastric (51%) or GEJ (49%) adenocarcinomas reported a response rate of 65%, median TTP of 8.3 mo, and median survival of 12.3 mo. Although the chemotherapy-related toxicity was as expected, the favorable efficacy results were counterbalanced by the following bevacizumab-related toxicities: two patients with a gastric perforation, one patient with a near perforation (overall incidence of perforation 6%), 25% incidence of grade Ⅲ or Ⅳ thromboembolic events, and 4% incidence of grade Ⅲ hemorrhages[42]. In a second, single-arm phase Ⅱ trial ($n = 42$) using a modified docetaxel, cisplatin, and fluorouracil (DCF) regimen in combination with bevacizumab in patients with metastatic gastric or GEJ adenocarcinoma, similar results for efficacy were observed. The incidence of grade Ⅲ/Ⅳ venous thromboembolism was 29%, where 93% of these thromboembolic events were asymptomatic and only identified on protocol-specific scans. One patient developed a gastrointestinal perforation[43]. Accordingly, gastrointestinal perforation and thromboembolic events may present a serious drawback for the use of bevacizumab in gastric cancer, indicating that a careful risk analysis is needed in randomized trials. Thus, based on these efficacy results, a randomized trial (AVAGAST) comparing capecitabine/cisplatin alone with capecitabine/cisplatin plus bevacizumab as a first-line therapy is currently being conducted on 760 patients with AGC. In a perioperative setting, another randomized trial is

also ongoing to compare ECX with ECX plus bevacizumab in the UK.

The multi-TKI, sunitinib, has also exhibited activity against VEGFRs, as well as Raf, platelet-derived growth factor receptor beta, fibroblast growth factor receptors, and c-KIT. At present, suntinib 50 mg/d as a single agent has been studied as a second- or third-line treatment for AGC in two nonrandomized phase Ⅱ studies[44,45]. Preliminary data from an Asian study (n = 42) showed a partial response rate of 5% and stable disease in 36% of the patients, plus sunitinib was well tolerated in these pretreated patients. Thus, a randomized trial of second-line chemotherapy and sunitinib *vs* a placebo is necessary to establish the therapeutic benefit of sunitinib in this pretreated patient population. Sorafenib is a potent inhibitor of the Raf tyrosine kinase, as well as several other receptor tyrosine kinases involved in the progression of gastric cancers, such as VEGFR-2 and VEGFR-3[46]. The median survival in a first phase Ⅱ study (n = 44) in patients with metastatic (80%) or locally advanced (20%) gastric and GEJ cancer using sorafenib (400 mg twice daily orally in combination with docetaxel and cisplatin in a 21 d cycle) was 14.9 mo, with progression-free survival at 5.8 mo and a response rate of 38.6%. Other phase Ⅱ studies using sorafenib combined with capecitabine or S-1 plus cisplatin are also currently being conducted in Korea and Japan.

Other targeting agents

Everolimus (RAD001) is an oral inhibitor of mTOR (mammalian target of rapamycin), which is downstream of the Akt pathway. After obtaining a remarkable response in patients with metastatic gastric cancer in previous phase Ⅰ / Ⅱ studies in Japan, a prospective randomized placebo-controlled study evaluating the efficacy of everolimus as a second- or third-line therapy in patients with AGC is now being conducted. A high level of c-Met expression has been correlated with the metastatic spread of tumors and poor survival in patients with various types of tumor, including gastric cancer[47], suggesting that it may be a suitable therapeutic target for gastric cancer. Therefore, several agents targeting c-Met are now in an early developmental stage, including the evaluation of MK2461, a TKI of activated c-Met, in a joint Korea-Japan study.

The development of ascites is a major clinical problem in patients with AGC, and the epithelial cell adhesion molecule (EpCAM), which has been shown to be highly overexpressed in gastric, as well as several other epithelial cancers, is the target for the trifunctional bispecific antibody, catumaxomab. The intraperitoneal administration of catumaxomab in patients with malignant ascites due to EeCAM-positive epithelial cancers resulted in a significantly increased puncture-free survival in a randomized study[48]. The side effects were mostly cytokine release-related symptoms (pyrexia, nausea, and vomiting) and abdominal pain, which were generally mild to moderate and fully reversible.

CONCLUSION

Many randomized clinical studies investigating cytotoxic chemotherapeutic agents have been conducted throughout the world, achieving some advances in the treatment of AGC. While no globally accepted standard regimen has yet been established, the combination of 5-FU and a platinum analog is still the most widely accepted reference regimen worldwide, although 5-FU can be replaced by capecitabine or S-1 and cisplatin by oxaliplatin.

Notwithstanding, emerging data from the clinical development of molecular targeted agents have provided novel opportunities that are expected to translate into survival benefits in the treatment of AGC. Recently, the final results of the ToGA study demonstrated that the addition of trastuzumab to combination chemotherapy can achieve remarkable survival advantages in patients with HER-2 positive AGC. However, this benefit is only limited to about 20% of patients with AGC (patients with HER-2 positive AGC). Therefore, there remains a critical need for both the development of more effective agents and the identification of molecular predictive and prognostic markers to select those patients who will benefit most from specific chemotherapeutic regimens and targeted therapies.

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BRIEF ARTICLE

Aberrant promoter methylation of *p16* in colorectal adenocarcinoma in North Indian patients

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Abstract

AIM: To investigate *p16* gene methylation and its expression in 30 patients with sporadic colorectal adenocarcinoma in a North Indian population.

METHODS: Methylation specific polymerase chain reaction was used to detect *p16* gene methylation and immunohistochemistry was used to study the p16 expression in 30 sporadic colorectal tumors as well as adjoining and normal tissue specimens.

RESULTS: Aberrant promoter methylation of *p16* gene was detected in 12 (40%) tumor specimens, whereas no promoter methylation was observed in adjoining and normal tissue. Immunohistochemistry showed expression of p16 protein in 26 (86.6%) colorectal tumors whereas complete loss of expression was seen in 4 (13.3%) and reduced expression was observed in 12 (40%) tumors. In the adjoining mucosa, expression of p16 was in 11 (36.6%) whereas no clear positivity for p16 protein was seen in normal tissue. There was a significant difference in the expression of p16 protein in tumor tissue and adjoining mucosa ($P < 0.001$). The methylation of the *p16* gene had a significant effect on the expression of p16 protein ($P = 0.021$). There was a significant association of methylation of *p16* gene with the tumor size ($P = 0.015$) and of the loss/reduced expression of p16 protein with the proximal site of the tumor ($P = 0.047$). Promoter methylation and expression of p16 had no relation with the survival of the patients ($P > 0.05$).

CONCLUSION: Our study demonstrated that promoter hypermethylation of the *p16* gene results in loss/reduced expression of p16 protein and this loss/reduced expression may contribute to tumor enlargement.

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Key words: Methylation specific polymerase chain reaction; p16; Methylation; Immunohistochemistry; Colorectal cancer

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INTRODUCTION

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in the Western world[1]. The incidence of CRC is very dynamic worldwide and many Asian countries have experienced a two- to four-fold increase in the incidence of CRC during the past few decades[2]. Adopting a Western-style diet high in fat and meat protein and low in fiber, vegetables, and fruit and a more sedentary lifestyle are believed to be the reasons underlying the increase. However, the interaction between these factors and the genetic characteristics of Asian populations might also have a pivotal role. CRC is one of the best-studied systems of multistage human carcinogenesis. The well-described model of colorectal carcinogenesis envisions a stepwise accumulation of early and late genetic alterations in the progression of adenoma to carcinoma[3]. It is becoming increasingly apparent that cancer arises through a series of not only genetic but also epigenetic alterations. The epigenetic mechanisms play a prominent role in CRC. Aberrant DNA methylation has long been noted in colorectal carcinogenesis; imbalances in methylation are thought to occur early in the process and are characterized by genome-wide hypomethylation and regional and locus-specific hypermethylation[4]. Among the common targets for aberrant DNA methylation is the *p16* gene[5,6]. *p16* is a gene which seems to play a major role in colorectal carcinogenesis. It is a tumor suppressor gene which is also known as *MTS-1*, *INK4a*, *CDKN2A*, and is composed of three exons, which encode 156 amino acids[7]. This gene is located on chromosome 9p21 and encodes a G1 cyclin-dependent kinase (CDK) inhibitor that can interrupt the tumor cycle by acting as a tumor suppressor gene and induces G1 cell cycle arrest[8,9]. Inactivation of the *p16* gene is now recognized as the second most common molecular defect in human cancer preferentially through *de novo* methylation of its 5'-promoter associated CpG Island[10,11].

In the present study, we looked the occurrence of *p16* gene promoter methylation and expression. We also explored the effect of *p16* gene methylation on protein expression in colorectal adenocarcinoma patients from India, in the cancer tissue, adjoining tissue and normal mucosa. The relationship of *p16* gene methylation and expression with clinicopathological parameters was also studied. To the best of our knowledge this is the first study in Indian population. The prevalence of adenoma is quite low in Indians and this raises a question whether the Indian population follows the same pattern of genetic alterations as in the west. Epigenetic alterations that suppress gene expression are a reversible phenomenon. So a detailed understanding of these alterations will provide insight into possible preventive strategies for colorectal carcinogenesis.

MATERIALS AND METHODS

Sample collection

Between July 2004 and July 2006, 30 patients meeting the selection criteria and diagnosed with colorectal adenocarcinoma undergoing surgery at the Postgraduate Institute of Medical Education and Research, India were prospectively included in this study designed to examine the promoter methylation and expression of the *p16* gene in normal colonic mucosa, adjoining and tumor tissues. Only patients undergoing resectional surgery were included. Patients who had history of prior chemotherapy or radiotherapy, with inoperable tumors, family history of colorectal adenocarcinoma, or those with mucinous/signet cell carcinoma were excluded from the study. Follow-up end point was September, 2007. A written informed consent was obtained from each patient for inclusion in this study, which was carried out after obtaining a formal approval from the Institute Ethics Committee. Apart from the description of the gross features of the tumor at the time of surgery, the rest of the colon was examined for any synchronous polyp or tumor. Fresh samples from tumor, adjoining (2-5 cm from the tumor) tissue and distant mucosa (5-10 cm from the main tumor mass) were taken from the resected colorectal specimen.

For histopathological analysis, freshly removed tissue samples were immediately fixed in 10% buffered formalin for 24 h, embedded in paraffin and histopathological assessment was carried out to determine the tumor grade and invasion. Fresh tissues were snap frozen within 10-15 min of surgical removal and stored at -80℃ until further use. Each tissue for the molecular analysis was also assessed histologically by making a crushed smear to verify the presence of tumors and only those samples, which contained > 90% of tumor cells were included for the final analysis. Similarly, the presence of adjoining and normal colorectal mucosa was also confirmed histologically before subjecting the tissue for further analysis. The normal mucosa was used as a control in each case.

Methylation analysis of the p16 gene promoter

All the specimens obtained during surgery procedure were treated with proteinase K and RNase. Each specimen was then subjected to DNA extraction using standard phenol-chloroform procedures[12]. The methylation status of 5' CpG islands of *p16* gene was assessed by bisulfite modification of DNA and methylation specific polymerase chain reaction (MSP) according to the method of Herman *et al*[13].

Bisulfite modification

Briefly, DNA (1 μg) in a volume of 50 μL was denatured by 0.2 mol/L NaOH for 10' at 37°C, then 30 μL of 10 mmol/L hydroquinone (Sigma, St. Louis, USA) & 520 μL of 3 mol/L Na bisulfite, pH 5.0 (Sigma, St. Louis, USA), both freshly prepared, were added to each sample. These were then incubated at 50℃ for 16 h. Modified DNA was purified using the Wizard DNA Purification Kit (Promega, Madison, WI, USA). Modification was completed by 0.3 mol/L NaOH treatment for 5 min at room temperature, followed by ethanol precipitation. DNA was resuspended in distilled water and stored at -20℃.

Methylation-specific polymerase chain reaction

The bisulfite modified DNA was polymerase chain reaction (PCR) amplified by using primers specific for methylated

CpG and unmethylated regions of the *p16* gene promoter. The PCR mixture contained 1 × PCR buffer (16.6 mmol/L ammonium sulphate / 67 mmol/L Tris, pH 8.8 / 6.7 mmol/L $MgCl_2$ / 10 mmol/L 2-mercaptoethanol), dNTPs (each at 2 mmol/L), 10 pmol of each primer (*p16* unmethylated sense: 5'GGTAGTTAGGAAGGTTGTATTGT3', *p16* unmethylated antisense: 5'TCCCTACTCCCAACCACA3', *p16* methylated sense: 5'TTGGTAGTTAGGAAGGTTGTATCGC3', *p16* methylated antisense: 5' TCCCTACTCCCAACCGCG3') and bisulfite treated DNA (approximately 50 ng) or unmodified DNA (50-100 ng) in a final volume of 25 μL. PCR specific for unmodified DNA also included 5% DMSO. Reactions were hot started at 95℃ for 5 min before the addition of 1.5 units of Taq DNA polymerase (Roche, GmbH, Germany). PCR amplification of the modified DNA samples consisted of 1 cycle of 95℃ for 5 min; 40 cycles of 95℃ for 30 s, 64℃ for 1 min, and 72℃ for 1 min; and 1 cycle of 72℃ for 5 min. PCR products amplified by unmethylated and methylated primers were 124 bp and 126 bp respectively.

Each PCR product was loaded onto a 2% Agarose gel, stained with ethidium bromide and visualized under UV illumination. DNA treated *in vitro* with CpG methylase MSssI (Sss1 methyltransferase, New England Biolabs, Ipswich, MA, USA) was used as a positive control for methylated alleles of this gene. Controls without DNA were performed for each set of PCR. Each MSP was repeated at least three times.

Immunohistochemistry

Immunohistochemical analysis was performed according to the avidin/biotin complex method using the Novocastra concentrated peroxidase detection system (Novocastra, Newcastle, UK). The specimens were fixed with 10% formalin, embedded in paraffin, cut into sections 5-μm in thickness and mounted on slides coated with poly-L-lysine. The sections were deparaffinized by heating at 60℃ for 30 min, treated with xylenes, and dehydrated in alcohol. Endogenous peroxidase was blocked with 0.03% H_2O_2 in methanol for 20 min. The antigenic sites were unmasked by means of pressure cooker treatment for 15 min in 10 mmol/L Citrate buffer (pH 6.0). The sections were then incubated with p16 primary antibody (BD Biosciences, CA, USA) at 1:50 dilution for 3 h at room temperature. Sections were further incubated with biotinylated universal secondary antibody (1:40 dilution) for 20 min followed by incubation with strept avidin horseradish peroxidase (1:40 dilution) for 20 min at room temperature. The slides were developed using 3-3' diaminobenzidine as the chromogen and counterstained with hematoxylin followed by mounting with DPX. The negative control in each case was served by omission of primary antibody. Inflammatory cells and reactive stromal cells served as positive internal controls for p16 staining

The immunohistochemistry results were scored by taking percentage positivity and intensity of staining into account. An intensity score of 0: no staining, 1: weak positivity, 2: moderate positivity and 3: strong positivity was given.

Statistical analysis

Data were analyzed using statistical analysis software. Pearson's χ^2 was performed to analyze the relationship between *p16* methylation status and expression and with each of the clinicopathological parameters. Mann-Whitney *U* test was performed to determine the relationship between the expression of p16 and each of the clinicopathological parameters. The overall survival (OS) and disease-free survival (DFS) were estimated by the Kaplan-Meier method and the Log rank test was used to evaluate the difference between survival of the patients with and without methylation and expression of the *p16* gene.

RESULTS

Clinicopathological features

There were 30 patients (20 males) with age range from 24-90 years (median age 56 years). All the patients were symptomatic at the time of diagnosis. Presentation include abdominal pain (n = 18), change in bowel habits (n = 17), rectal bleeding (n = 15), and loss of appetite (n = 12). The other signs and symptoms were subjective weight loss (n = 14), abdominal mass (n = 6), vomiting or abdominal distention (n = 4), anemia (n = 5).

The distribution of tumor was 10% (n = 3) in cecum, 16.6% (n = 5) in ascending colon, 3.3% (n = 1) in transverse colon, 3.3% (n = 1) in hepatic flexure, 6.6% (n = 2) in splenic flexure, 10% (n = 3) in descending colon, 33.3% (n = 10) in sigmoid colon, and 16.6% (n = 5) in the rectum. Thus 18 (60%) patients had the tumor in the distal colon, and 12 (40%) in the proximal colon. None of the patients had a synchronous adenoma or carcinoma. The median length of the tumors was 5 cm (range 2-10 cm). According to the classification of International Union Against Cancer[14], there were 4 patients (13.3%) in stage Ⅰ, 18 (60%) in stage Ⅱ, 6 (20%) in stage Ⅲ and 2 (6.6%) in stage Ⅳ disease. Metastasis was found in 8 patients with distant metastasis in liver (n = 2) and lymph nodes (n = 6). None of the patients had a family history of CRC or any other kind of malignancy. All tumors were adenocarcinomas and on histological examination 6 were well differentiated, 21 moderately differentiated and 3 poorly differentiated adenocarcinomas.

Methylation analysis of p16 promoter in surgical specimens

Methylation status of the *p16* gene promoter was evaluated in colorectal tumors, adjoining and normal mucosa using MSP. All the surgical specimens showed the presence of a band of unmethylated DNA that could be derived from unmethylated DNA of normal, adjoining mucosal cells and tumor cells as well as normal constituents in the stroma such as vascular endothelial cells, smooth muscles, fibroblasts and inflammatory cells. A band of methylated DNA was found in 12 (40%) tumors whereas no methylation was observed in adjoining and normal mucosa (Figure 1).

Expression of p16 protein in surgical specimens

Immunohistochemistry was performed to evaluate pos-

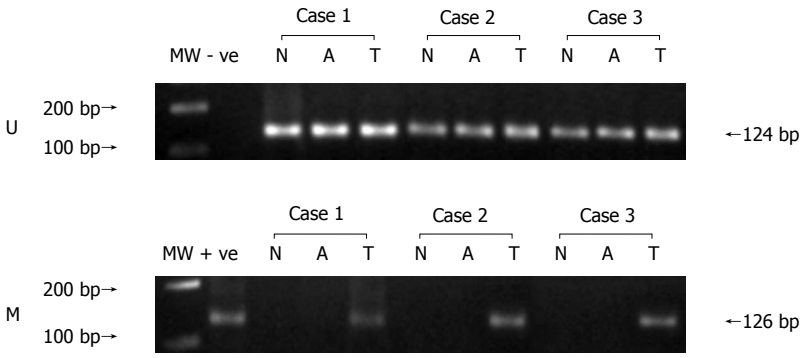

Figure 1 Methylation specific polymerase chain reaction for *p16* gene in colorectal tumor tissue, adjoining and normal mucosa. U: Polymerase chain reaction (PCR) with primers for unmethylated *p16*; M: PCR with primers for methylated *p16*; MW: Molecular weight marker; - ve: negative control (without template); + ve: Normal lymphocyte DNA completely methylated with CpG methylase (M.sss1) used as a positive control for methylation; N: Normal mucosa; A: Adjoining mucosa; T: tumor tissue.

sible differences in the expression of p16 protein in tumor tissues, adjoining and normal mucosa. No clear positivity of p16 protein was seen in majority of normal mucosa samples except for a few samples (13.3%), which showed surface nuclear positivity for p16 protein. In contrast, p16 was abundantly expressed in the majority of adjoining mucosa and CRC tissues and was localized both in the nuclei and cytoplasm. In adjoining mucosa 11 (36.6%) samples showed expression of p16 protein. The p16 expression was weak in 6 (20%) and moderate in 5 (16.6%) samples. In the case of tumors, p16 was expressed in 26 (86.6%) samples whereas complete loss of expression was observed in 4 (13.3%). The expression was reduced or weak in 12 (40%), moderate in 10 (33.3%) and strong in 4 (13.3%) tumors (Figure 2A-D). There was a significant difference between the expression of p16 in tumor and adjoining mucosa (131.12 ± 91.55 *vs* 42.80 ± 57.21, $P = 0.001$).

Effect of gene methylation on protein expression

The *p16* gene methylation was compared to the p16 protein expression and the results are summarized in Table 1, showing actual staining intensities of each specimen. The staining intensities were categorized into 3 groups 0-100, 100-200 and 200-300 intensity score. Out of the 26 colorectal adenocarcinoma samples positive for p16 protein expression, 8 had methylation in the promoter region. In the 8 methylated samples, 6 had reduced expression and 2 had moderate expression. However, 4 tumors totally negative for p16 expression also had methylation in their promoter region. There was a significant correlation of methylation of *p16* gene with the loss/reduced expression (the samples with staining intensity between 0-100) of p16 protein ($P = 0.021$, Table 2).

Relationship of p16 methylation and expression with clinicopathological parameters

The relationship between the methylation and level of staining of p16 in all the surgical specimens and several clinicopathological parameters was examined. These parameters included gender, tumor size, tumor site, histological grade, lymph node metastasis, distant metastasis, TNM staging, age, pre and post-operative serum CEA levels *etc*. A significant correlation was observed between methylation of *p16* gene and the size of the tumor ($P = 0.015$) and of the nuclear positivity of p16 protein with the proximal site of the tumors ($P = 0.047$). There was no correlation between *p16* gene methylation and protein expression with other clinicopathological parameters.

Survival analysis

Follow up data were available for 29 patients. At last follow-up (September, 2007), four patients (13.3%) had died, 4 patients (13.3%) showed the recurrence of disease with distant metastasis in 2 patients (6.6%) and 21 patients (70%) were alive without disease. The association of methylation and expression of p16 with DFS and OS was analyzed. DFS was defined as the time from the date of surgical resection of the tumor to the date of recurrence of the disease and OS was defined as the time from the date of diagnosis of CRC to the date of last follow up. The median follow-up period was 27 mo with a range of 8-39 mo (mean=25.5 ± 8.14 mo). By Kaplan-Meier log-rank survival analysis, survival of the patients with colorectal adenocarcinomas was not associated with the methylation and expression of p16 ($P > 0.05$; Figure 3).

DISCUSSION

CRC is a major cause of cancer death worldwide. The usual treatment is surgery and subsequent chemotherapy and radiotherapy. Many Asian countries have experienced two- to four-fold increase in the incidence of CRC during the past few decades. However, there are no such data from India. So it is important to determine genetic alterations in this cancer as an approach to predicting the malignancy of disease in the Indian population. Development of CRC is a multistep process which includes the involvement of various oncogenes and tumor suppressor genes. Several tumor suppressor genes contain CpG islands in their promoters, a fact which has prompted many workers to investigate the role of methylation in silencing these genes. DNA is methylated only at cytosine located 5' to guanosine in the

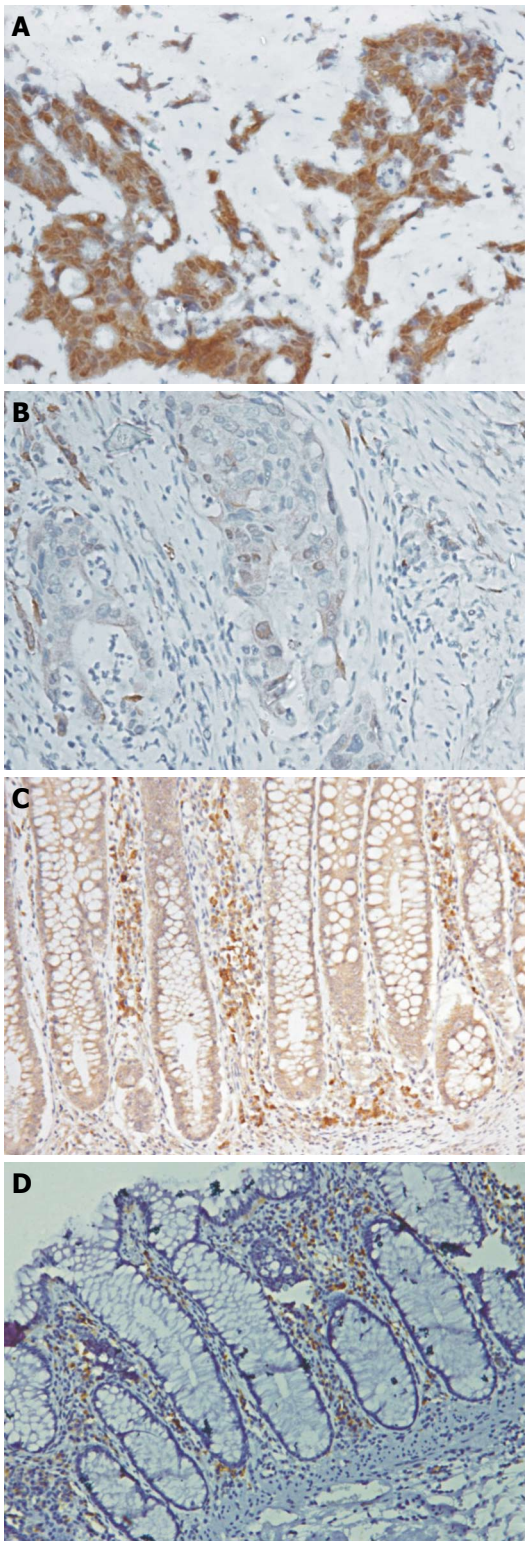

Figure 2 Immunohistochemical staining showing expression of p16 protein. A: Tumor showing strong nuclear and cytoplasmic positivity of p16; B: Tumor showing reduced cytoplasmic positivity for p16; C: Adjoining mucosa showing moderate cytoplasmic positivity for p16; D: Normal colorectal mucosa showing no positivity for p16 (SABP immunostaining; × 450).

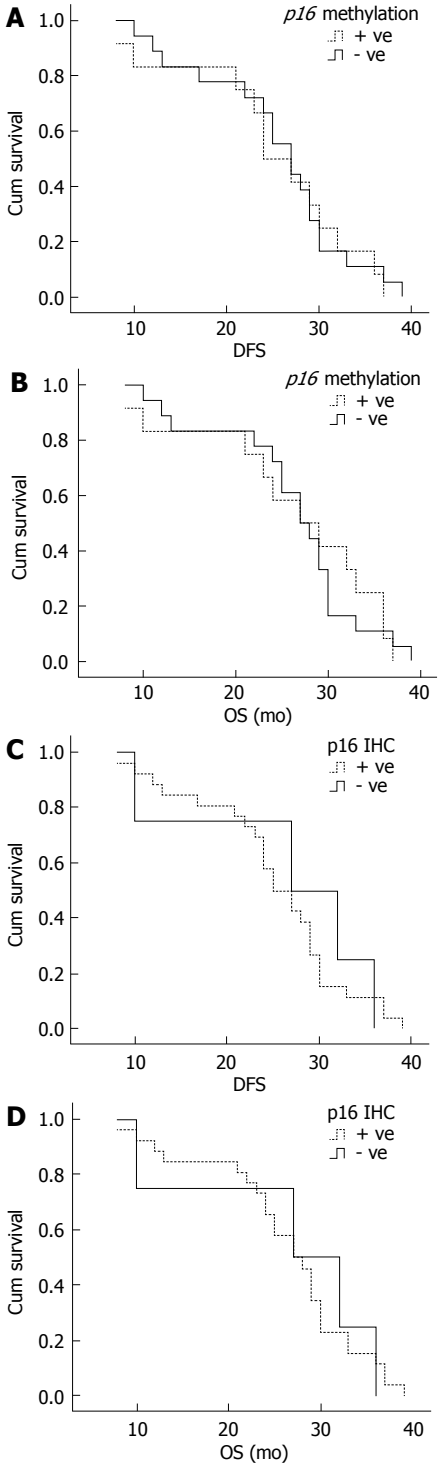

Figure 3 Survival curves of patients with colorectal adenocarcinoma showing correlation. A: *p16* methylation status with probability of disease-free survival (DFS); B: *p16* methylation status with probability of overall survival (OS); C: p16 protein expression with probability of DFS; D: p16 protein expression with probability of OS.

CpG dinucleotide. This modification has important regulatory effects on gene expression *via* blocking transcriptional activation[15]. *p16* is a tumor suppressor gene which plays an important role in the transition of cells through G1 to S phase of the cell cycle by binding to CDK 4 and inhibiting its binding to cyclin D1[16-18]. Methylation of cytosine residues at CpG sites in the *p16* gene promoter, resulting in silenced p16 expression, has been reported in many cell lines, including CRC, and some primary carcinomas of varied origins, such as colon, brain, breast, bladder, ovary, lung, and myeloma[19-32]. The rates of homozygous dele-

Table 1 Comparison of p16 expression to the *p16* gene methylation

Sample No.	Tumor		Adjoining		Normal	
	p16 protein expression	*p16* methylation	p16 protein expression	*p16* methylation	p16 protein expression	*p16* methylation
S-1	90	+	50	-	0	-
S-2	0	+	0	-	0	-
S-3	235	-	0	-	0	-
S-4	100	+	0	-	0	-
S-5	50	+	0	-	0	-
S-6	200	+	100	-	0	-
S-7	150	-	0	-	60	-
S-8	100	-	0	-	0	-
S-9	180	+	0	-	0	-
S-10	225	-	120	-	0	-
S-11	200	-	0	-	0	-
S-12	80	-	0	-	0	-
S-13	70	+	0	-	0	-
S-14	300	-	100	-	0	-
S-15	115	-	-	-	0	-
S-16	90	-	40	-	0	-
S-17	50	+	40	-	0	-
S-18	0	+	0	-	0	-
S-19	0	+	0	-	0	-
S-20	50	+	0	-	30	-
S-21	200	-	0	-	0	-
S-22	40	-	0	-	50	-
S-23	110	-	0	-	0	-
S-24	200	-	120	-	0	-
S-25	40	-	0	-	0	-
S-26	0	+	0	-	0	-
S-27	60	-	30	-	0	-
S-28	180	-	100	-	0	-
S-29	300	-	180	-	70	-
S-30	120	-	100	-	0	-

tion and intragenic mutation of the *p16* gene in CRCs are very low. Therefore, in the present study, methylation of 5' CpG islands of the *p16* gene in tumors, adjoining and normal colonic mucosa was examined by using the PCR-based methylation assay. The prevalence of *p16* hypermethylation in colorectal tumors, reported in different studies, varies according to the technique used. In the present study MSP was used. The percentage of hypermethylation of the *p16* gene in our study was 40% whereas, it ranged from 32%-37% in European[23,33],19%-36% in US[34-36] and 29%-42% in Asian population[37,38]. The percentage of *p16* methylation in CRC observed in our study fell within the range of those quoted in the literature. A band of unmethylated DNA was visible in each specimen. Similar results were also reported by other investigators[34]. This genetic aberration was found to be significantly correlated with the size of the tumor but not with the other clinicopathological parameters examined. Tumors > 5 cm exhibited *p16* gene methylation more than tumors ⩽ 5 cm suggesting that *p16* methylation may contribute to tumor enlargement in CRC.

p16 has been shown to be expressed in colorectal adenocarcinoma in some studies[39-41]. The reported percentage of expression has varied, with a majority of studies showing p16 expression in more than three quarters of CRC. In our study, p16 expression was observed in 86.6% of tumors whereas complete loss of expression was observed in 13.3% and reduced expression in 40% of tumors. These observations are in agreement with the study of Kim *et al*[42] who have shown that the majority of CRCs (96.4%) over expressed p16 protein. Similarly, Lam *et al*[43] reported p16 expression in 78% of the tumors. A study by Palmqvist *et al*[44] reported that only 18% of CRCs were classified as p16 negative, whereas the majority (82%) exhibited p16 positivity. The expression of p16 was more frequently noted in proximally located tumors (P = 0.047). The difference between proximal and distal colorectum in p16 expression could account for their different clinical behavior. The high frequency of p16 expression in tumors and the absence of p16 in non tumor epithelium implies that the *p16* aberration is an important step in the pathogenesis of CRC.

There was a significant correlation between methylation and loss or reduced expression of p16 in this study (P = 0.021). *p16* methylated tumors showed either loss of expression or reduced expression. Most studies have suggested that detectable *p16* gene methylation is necessarily linked to the inactivation of p16 protein or transcriptional silencing of *p16* gene[10,45-48]. Coexistence of *p16* gene methylation and p16 expression in the same specimen has also been frequently described[39,48,49] and this might reflect the cell heterogeneity, in which some cells contained showed *p16* gene methylation and loss of p16 expression whereas others expressed or even overexpressed p16 protein. Ohhara *et al*[50] have proposed that activation but not inactivation of the *p16* gene was associated with primary CRC. In the present study, we found that the methylation

Table 2 Comparison of p16 protein expression with *p16* gene methylation

			p16 expression (IHC score)			Total
			0-100	100-200	200-300	
p16 Methylation	- ve	Count	6	8	4	18
		% within p16 expression	33.3%	44.4%	22.2%	100.0%
		Count	10	2	0	12
Total	+ ve[1]	% within p16 expression	83.3%	16.6%	0.0%	100.0%
		Count	16	10	4	30
		% within p16 expression	53.3%	33.3%	13.3%	100.0%

[1]$P = 0.021$, Pearson's χ^2 test.

of the *p16* gene results in loss or reduced expression of p16 protein, but the overall percentage of expression of p16 was high, whereas majority of the samples showed reduced p16 expression. The reduced expression of p16 in some tumors lacking methylation suggested that not only the methylation but some other genetic alterations are responsible. This other genetic alteration could possibility be mutation in the coding region of *p16* gene which could also reduce the expression of p16 protein in tumors lacking methylation. Preliminary observation on the same CRC specimens indicate that 20% of the samples of CRC showed mutation in exons 2 and 3 of the coding region of *p16* gene, although more comprehensive analysis of the site and nature of mutation is required. The overexpression of p16 protein in some of the tumors lacking methylation indicated that the activation, but not the inactivation, of the gene was associated with the overexpression and tumor progression. Taken together these results suggest that *p16* hypermethylation may, at least in part, contribute to reduced expression of p16. The elucidation of the relationship between p16 expression and *p16* gene methylation in primary tumors requires further studies on a large number of samples and this may certainly help us to better understand the role of methylation of tumor suppressor genes in carcinogenesis.

There are not many reports in the literature evaluating the prognostic role of *p16* hypermethylation and expression. Some investigators have shown that hypermethylation of the *p16* gene was associated with advanced tumor stage and shorter survival[37,38]. In our patients no correlation could be established between the methylation and expression of p16 and survival.

All reported studies have assumed the adenoma→carcinoma sequence and reported the presence of genetic alterations in adenomas, a precursor lesion that finally develops to carcinoma. However, only a very small proportion of the Asian patient population has develop adenomas. The present study, therefore included adjoining mucosa in order to determine the initial changes in CRC. No methylation was observed in the adjoining mucosa, suggesting that there were no changes near the tumor region and that the process of tumorigenesis is restricted to a limited area. This is the first study in which we analyzed both the adjoining and normal mucosa to determine the initial changes in CRC.

This is the first study in the Indian population in which *p16* gene has been analyzed at both genetic and expression level in CRC in relation to clinicopathological features and prognosis. The frequency of alterations of this gene in this cohort is similar to others. This shows that despite the rare occurrence of synchronous adenomas in the population studied, the frequency of alterations in this gene is almost same as observed in other studies, suggesting that the process of tumorigenesis is similar overall although there is a difference at the initiation stage. This study is the first one in an Indian population. It was limited to by the size of the cohort and confined to North India. We need more data from the other parts of the country to validate our findings

In conclusion, our study demonstrated that majority of CRC tissues expressed p16 protein and that the low or reduced expression of p16 among CRC tissues, probably caused by hypermethylation, may contribute to tumor enlargement.

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COMMENTS

Background

Colorectal cancer (CRC) is a worldwide health problem. The incidence of CRC is very dynamic worldwide and during the past few decades there has been a dramatic increase in the incidence of CRC in Asian countries although the exact cause of this increase is not known. Therefore, this study was planned to investigate the molecular genetic alterations responsible for the development of CRC in the Indian population. *p16* is a cell cycle regulator and inactivation of this gene leads to uncontrolled cell proliferation and growth. The inactivation of this gene is mainly associated with aberrant promoter methylation. Therefore in the present study the authors analyzed the promoter methylation and expression of the *p16* gene in colorectal adenocarcinoma in Indian patients.

Research frontiers

p16 is a major genetic marker in the development of colorectal carcinogenesis. It is well studied in various populations but there are no previous data from Indian population.

Innovations and breakthroughs

p16 gene hypermethylation may at least in part contribute to reduced/loss of expression of p16 protein. This is the first study in an Indian population and for the first time *p16* has been analyzed at both genetic and expression levels in CRC in relation to clinicopathological features and prognosis.

Applications

p16 plays a very important role in the development of colorectal carcinogenesis. Inactivation of the gene due to promoter methylation leads to lost or reduced

gene function. Hypermethylation is a reversible phenomenon. The identification of these genetic markers are implicated in colorectal carcinogenesis at its early stages can be very helpful for treating the patients with CRC.

Peer review

This study examines *p16* promoter methylation using methylation-specific PCR in resected tumors from a cohort of 30 colon cancer patients in North India. *p16* methylation in matched adjacent tumor and normal tissue is also examined. p16 expression was determined by IHC score. This paper is appropriate and well written.

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CASE REPORT

Large ulcerated cecal lipoma mimicking malignancy

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Abstract

Colonic lipomas are relatively uncommon tumors of mesenchymal origin, composed of well-differentiated adipose tissue supported by fibrous tissue, that usually occur in cecum and ascending colon. Colonic lipomas rarely cause symptoms and are usually detected incidentally. However, if the lesion is large, it may produce symptoms, such as abdominal pain, rectal bleeding, obstruction, intussusception, and even weight loss. Large colonic lipomas can be mistaken for malignancy, which may result in extensive surgical operations. We report a large broad-based ulcerated cecal lipoma in a 68-year-old woman, who presented with abdominal pain and weight loss. The ulcerated lesion was highly suspicious for malignancy radiologically and endoscopically. The patient underwent laparoscopic right-hemicolectomy, and the lesion was diagnosed as a cecal submucosal lipoma. The surgical approach remains the treatment of choice for large and complicated cases.

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Key words: Cecum; Lipoma; Carcinoma

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INTRODUCTION

Lipoma of the colon is a rare condition which may be detected incidentally at colonoscopy, surgery or autopsy[1]. Most colonic lipomas occur in the cecum and ascending colon, and are asymptomatic and need no treatment. However, if the lesion exceeds 2 cm in diameter, it may produce symptoms, such as abdominal pain, bleeding, obstruction, intussusception, and even weight loss[2-6]. Large colonic lipomas, can sometimes be mistaken for malignancy, which may result in extensive surgical operations[7]. We report a large broad-based ulcerated cecal lipoma (6.0 cm in greatest dimension) in a 68-year-old woman, who presented with abdominal pain and weight loss. The ulcerated lesion was highly suspicious for malignancy radiologically and endoscopically. The patient underwent laparoscopic right hemicolectomy, and the lesion was diagnosed as a cecal submucosal lipoma pathologically.

CASE REPORT

A 68-year-old woman with history of hypertension, coronary artery disease, hypercholesterolemia, gastroesophageal reflux disease, and arthritis, presented with abdominal pain for 1 mo and 10 pounds weight loss within 7 mo. The patient noted that the pain was in the right abdomen with a gradual onset and it was relieved after passing stool. The pain was 5/10 on the pain scale. The patient had no constipation or bloody stool. Physical examination of the abdomen revealed no masses or tenderness. Stool examination was negative for occult blood. The hemoglobin level was 10.6 g/dL (normal: 12.0-16.0 g/dL). Serum carcinoembryonic antigen level was within normal limits. Helical computed tomography (CT) imaging of the abdomen

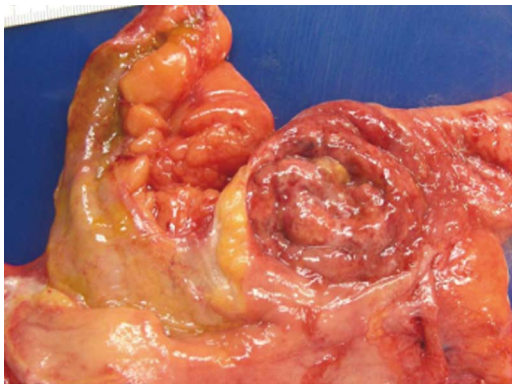

Figure 1 Gross photograph of broad-based and ulcerated mass in cecum (6.0 cm in greatest dimension) mimicking malignancy.

and pelvis with intravenous and enteric contrast showed a cecal mass extending into the lumen with marked wall thickening, and multiple subcentimeter lymph nodes in the abdominal right lower quadrant. The CT diagnosis was that inflammatory *vs* neoplastic process could not be differentiated, and correlation with colonoscopy and biopsy were recommended. The following colonoscopy examination revealed an ulcerated mass located in the cecum, and the colonoscope could not pass beyond the lesion that appeared grossly malignant. Multiple biopsies from the lesion were obtained and sent for pathology. Histopathologic examination of biopsy specimens showed colonic mucosa with areas of acutely inflamed fibrous exudates and no identifiable malignancy.

Because of the radiological and endoscopical suspicion of malignancy and even though no malignancy was identified in biopsy specimens, the patient underwent laparoscopic right hemicolectomy. Pathological gross examination showed a cecal ulcerated mass with a broad base measuring 6.0 cm × 5.1 cm (Figure 1). The ulcerated surface was covered with green/grey necrotic and mucinous material. Cut sections showed the ulcerated lesion was within the submucosa with a tan-yellow appearance and soft consistency. The lesion had no clear border with the surrounding tissues. Histopathologic examination revealed that the mass was located in the submucosa and composed of mature adipose tissue. The adipose tissue had no demarcated border to the surrounding tissue, and even protruded into the adjacent submucosa which was covered by chronic inflamed cecal mucosa (Figure 2A and B). The overlying mucosa was ulcerated and replaced by granulation tissue with acute and chronic inflammatory cells infiltration (Figure 2C). There was no carcinoma identified. The pathology diagnosis was cecal submucosal lipoma with overlying mucosa ulceration.

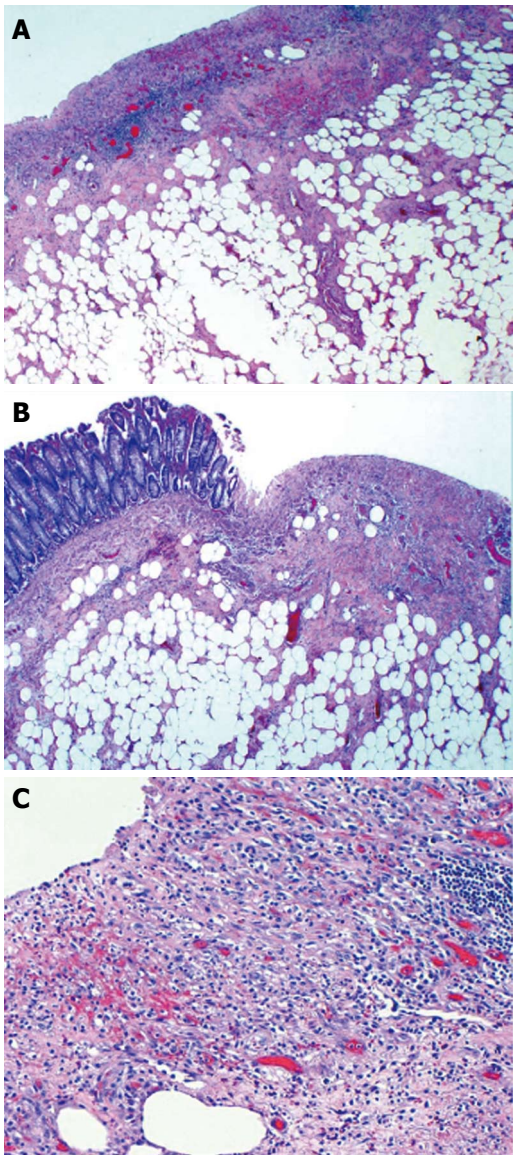

Figure 2 Histopathologic examination. A: Submucosal mass is composed of mature adipose tissue covered by granulation tissue with inflammatory cells infiltration (HE stain, × 100); B: Junction of ulcerated mass and adjacent cecal mucosa (HE stain, × 100). The submucosal lipoma has no defined margin to the surrounding tissues. The cecal mucosa presents with chronic inflammation; C: Overlying cecal mucosa replaced by granulation tissue with acute and chronic inflammatory cells infiltration (HE stain, × 400).

DISCUSSION

Lipomas are rare nonepithelial benign tumors of the gastrointestinal tract, and are found most frequently in the cecum and ascending colon. Weinberg and Feldman reviewed 1310 autopsies and found an incidence of 4.4%[8]. However, a meta-analysis by these same investigators identified 135 gastrointestinal lipomas in over 60 000 autopsies, with an incidence of 0.2%[8]. Colonic lipomas arise in the submucosa but occasionally extend into the muscularis propria or subserosa[3,9,10]. Usually, the lipomas are solitary, but can be multiple[11]. A rare lipomatosis syndrome, with numerous lipomas throughout the bowel, has been described[12,13]. Colonic lipomas usually do not cause symptoms and are often discovered incidentally at colonoscopy, surgery or autopsy. Only 25% of patients with colonic lipoma develop symptoms. When lipomas are larger than 2 cm in diameter they may cause symptoms including abdominal pain, diarrhea or constipation, weight loss, bowel obstruction either secondary to intussuseption or through direct luminal protrusion of the enlarging mass. Rarely, ulceration of the overlying mucosa may cause clinically apparent bleeding or chronic anemia[2-6].

Colonic lipomas are mainly found in the ascending colon and cecum, and typically appear grossly as small polypoid masses with a wide base and normal overlying mucosa[3,14].

The incidence of lipomas relative to all polyploid lesions of the large intestine is reported to range from 0.035% to 4.4%[15]. Various imaging modalities can indicate the diagnosis of colonic lipomas[3,4,14]. In spite of this, colonic lipomas continue to present difficulties in the preoperative differentiation between malignant and benign colonic neoplasm. Barium enema may reveal an ovoid filling defect with well-defined borders. A so-called "squeeze sign", indicating a change in size and shape of a radiolucent lesion in response to peristalsis or the application of external pressure to the abdomen, can sometimes be elicited. CT scan may demonstrate a well-circumscribed intraluminal mass with absorption densities characteristic of fatty tissue. In the current case, CT scan did not show the typical features of lipoma. This was probably due to the large ulcerated mass with a broad base, unclear border to the surrounding tissues, and granulation tissue formation, which are different from the typical polypoid mass containing fatty tissue and protruding into the lumen. Endoscopy can usually distinguish lipomas from cancer or other tumors. Lipomas are seen as smooth, rounded yellowish polyps with a thick stalk or broad-based attachment. Typical colonoscopic features are the "cushion sign" or "pillow sign" (pressing forceps against the lesion results in depression or pillowing of the mass) and the "naked fat sign" (extrusion of yellowish fat at the biopsy site)[4]. Usually, the mucosa overlying a colonic lipoma is intact. In rare cases, as in the present patient, colonoscopy may reveal large-sized flat-shaped mass with ulceration that may lead to a impression of malignancy[7].

The etiology of gastrointestinal lipomas is not yet well understood[16]. In the majority of cases they are true neoplastic lipomas. They arise from the submucosa with a well-circumscribed margin, and protrude into the gastrointestinal lumen. They have a polypoid appearance, and rarely extend to muscularis propria, even subserosa. However, sometimes, the lipoma may be a result of chronic inflammation, especially in the cecum. The chronic inflammatory process may result in abnormal intestinal motility and this may cause the mucosa to pull away from the deeper submucosa, resulting in the creation of a tissue space with subsequent adipose tissue deposition. The deposited adipose tissues have no well-defined margins with adjacent tissues, and the overlying and adjacent colonic mucosa always presents with inflammatory changes, as in our current case. In this situation, it is better to call this a "pseudolipoma" to distinguish it from the true neoplastic lipoma. However, the differentiation of neoplastic lipoma and pseudolipoma is still controversial and not well recognized.

Generally, small colonic pedunculated lipomas can be safely removed endoscopically. Endoscopic resection of large colonic lipomas remains controversy. Surgical resection is recommended for larger lipomas to relieve the symptoms or exclude malignancy. Colostomy with lipomectomy and limited colon resection are considered an adequate treatment for certain lipomas diagnosed preoperatively. A segmental resection, hemicolectomy or subtotal colectomy may be necessary in cases when diagnosis is questionable or when a complication occurs. If the preoperative diagnosis of colonic lipoma can be made correctly, the extent of surgery may be appropriately limited[17,18].

In conclusion, colonic lipomas are rare nonepithelial benign tumors. Their accurate preoperative diagnosis is difficult and they can be mistaken for malignancy, especially when the lesion is large in size, and with ulceration. The etiology can be that of a true neoplasm or that of an inflammatory process. A surgical approach remains the treatment of choice for large and complicated cases.

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CASE REPORT

Mucinous carcinoma in Crohn's disease originating in a fistulous tract

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Abstract

Malignant disease, including mucinous carcinomas of the colorectum, may complicate long-standing Crohn's disease. An 18-year-old male with extensive small and large bowel involvement with Crohn's disease developed recurrent peri-rectal fistulous disease that persisted for more than a decade despite pharmacological and surgical therapy as well as later therapy with biological agents. Eventually, an extensive and difficult-to-detect mucinous carcinoma developed in the fistulous tract. Although fistula cancer is rarely described in Crohn's disease, use of immunosuppressant and biological agents may play an initiating or exacerbating role in its development or progression. As potent biological agents are frequently used, often to avoid surgical treatment, clinicians should have an increasingly high index of suspicion for this potential complication, especially if fistulous drainage persists and remains refractory to medical therapy.

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Key words: Tumor necrosis factor antibodies; Anorectal adenocarcinoma; Crohn's disease; Infliximab; Adalimumab; Anal fistula; Fistula carcinoma

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INTRODUCTION

Infliximab, a mouse-human chimeric monoclonal antibody to tumor necrosis factor α (TNF-α), appears to reduce disease activity in Crohn's disease[1,2]. Fistula drainage can also be reduced or terminated, but the role of biological agents in causing complete resolution of fistulous tracts remains controversial[3,4]. Adalimumab, a more "humanized" monoclonal antibody to TNF-α, has also been used in Crohn's disease. Soon after the introduction of these agents, reports of serious suspected adverse effects began to appear[5,6]. Concern has been expressed for the potential to increase risk for malignant disease possibly reflecting, in part, *in vivo* biological effects of anti-tumor necrosis factor agents[7]. Hepatosplenic lymphoma, a rare T-lymphocyte malignant disorder, has been reported in children, after treatment with infliximab[8-10].

Cohort and population-based studies have also described an increased intestinal cancer risk in Crohn's disease[11-13]. Other neoplasms may also occur more frequently in Crohn's disease, including myeloid and lymphoid malignancies as well as carcinoid tumors[14-16]. Characteristics of the colorectal cancers in Crohn's disease appear to include: long-standing and extensive colonic disease, young age at cancer diagnosis, tendency to localize in the distal colorectum and mucinous type histopathogical features[12].

In Crohn's disease, concern has been expressed regarding a potential for superimposed cancer risk associated with use of biological agents, particularly with fistula closure[17].

CASE REPORT

An 18-year old male was first seen in November 1995 with abdominal pain and diarrhea, present for 3 years. Physical exam was normal. Fecal studies for occult blood, bacteria and parasites were negative, but fecal leukocytes were present. Lab studies showed an anemia (hemoglobin, 110 g/L *vs* normal, 130-172 g/L) with an elevated sedimentation rate to 38 mm/h and a reduced serum albumin of 30 g/L (normal, 35 to 50). Upper gastrointestinal barium series, including a small bowel follow through, revealed 4 short segments of narrowing from the distal jejunum to ileum, including terminal ileum, consistent with Crohn's disease. Treatment with a 5-aminosalicylate (5-ASA) alone led to symptom resolution.

Two years later, diarrhea recurred with weight loss of 4 kg, in spite of 5-ASA. Physical exam showed an anal fissure. Hemoglobin was 98 g/L and sedimentation rate was 72 mm/h. Serum albumin was 21 g/L. Flexible sigmoidoscopy and biopsy showed focal aphthoid ulcers with inflammatory changes but no granulomas. The patient was treated with a 6-wk course of prednisone. As his symptoms resolved, he discontinued 5-ASA.

In November 1997, a perianal abscess developed and required surgical drainage. Because of diarrhea and further weight loss of 5 kg, parenteral nutrition was given. A colonoscopy and biopsies were normal. Although the patient's diarrhea resolved and weight improved with 5-ASA and oral budesonide, anorectal pain and drainage persisted so ciprofloxacin and metronidazole were added. Examination under anesthesia revealed that a fistulous tract extended 6 to 10 cm cephalad and a sinogram showed extension into the ischiorectal space. After further surgical drainage and packing, he was treated with metronidazole for 3 mo. His symptoms resolved and blood tests normalized. A small bowel barium study was reported to be normal.

One year later, abdominal pain recurred and a small bowel barium study showed numerous strictures. The patient was unable to eat because of pain from obstructive symptoms, so parenteral nutrition was provided. At laparotomy, 30 cm of the mid-jejunum and 35 cm of the ileocecum were resected and 7 stricturoplasties performed. Resected small bowel showed transmural inflammation, focal acute and chronic inflammation, ulceration and fibrosis consistent with Crohn's disease. After surgery, his appetite and bowel function became normal and he regained his weight.

In March 1999, anorectal pain and drainage recurred that failed to respond to ciprofloxacin and metronidazole. An ischiorectal abscess was drained. Despite ongoing ciprofloxacin and metronidazole, the incisional site continued to drain intermittently. In March 2000, anorectal pain recurred along with rectal bleeding. Colonoscopy revealed limited patchy areas of focal aphthoid ulceration in the sigmoid and descending colon. Biopsies showed inflammatory change with a single well-formed granuloma. Because of paraesthesiae in his feet, metronidazole was discontinued. The possibility of ileostomy or diverting colostomy was offered, but he declined further surgery. The patient requested referral for infliximab treatment.

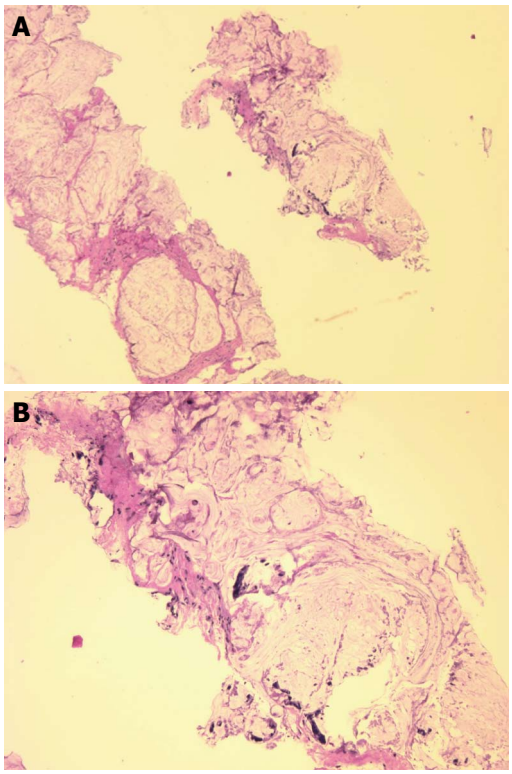

Figure 1 Needle biopsy fragments showing mucinous adenocarcinoma (HE stain). A: Low power photomicrograph above shows fragments of infiltrating cancer; B: High power photomicrograph below shows individual cancer cells or clusters of cancer cells within mucus.

Over the next year, the patient was treated with 5-ASA, budesonide, ciprofloxacin and metronidazole. In addition, azathioprine and prednisone were given. In spite of these medications, his perianal fistula continued to drain. Infliximab infusions were initiated and provided up to 2007, along with 5-ASA and azathioprine. In October 2006, despite infliximab therapy, another ischiorectal abscess was drained. Unfortunately, persistent drainage occurred while left lower quadrant and perianal pain developed with radiation into his right lower limb. Endoscopic examination showed no rectosigmoid abnormality. In November 2007, subcutaneous adalimumab was tried but his pain worsened and drainage continued.

Magnetic resonance imaging (MRI) revealed a large right-sided perirectal mass and CT guided core biopsies confirmed the presence of mucinous adenocarcinoma from the fistulous tract (Figure 1). The lesion also extended inferiorly into the pelvic floor and perineum, anteriorly into the prostate gland and seminal vesicles, and posteriorly into the lumbar spine (Figure 2). The patient underwent a diverting loop colostomy, followed by local radio-therapy. Post-operatively, he required palliative treatment for ongoing severe pain and, in July 2008, he succumbed to the extensive malignancy at age 28 years.

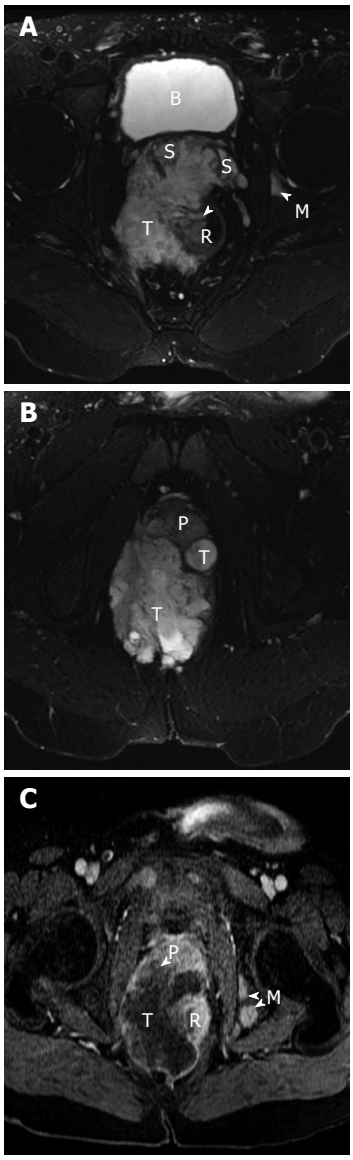

Figure 2 Magnetic resonance images of the patient. A and B show the axial T2-weighted fat suppressed MR images of the pelvis. A heterogeneous but predominantly high signal intensity tumor mass (T) is seen arising from the right lateral wall (arrow) of the rectum (R). This mass extends anteriorly to invade both seminal vesicles (S) and abuts and displaces the prostate gland (P). A high signal focus in the left acetabulum represents a bone metastasis (M). The bladder is noted as B. C is the MR image about 5 wk later, now repeated with contrast. T1-weighted post-contrast fat saturation MR image correlating to image in Figure 2b shows rim enhancement of the mass with a large central low signal area consistent with central necrosis. There is now invasion of the tumor into the prostate gland (P) and multiple new osseous metastases (M).

DISCUSSION

This report describes Crohn's disease in a young man involving the small and large intestine, complicated by recurrent peri-anal and peri-rectal fistulous disease. Both drug and surgical treatment of the fistula were attempted; eventually, biological agents were used. Although symptomatic improvement temporarily occurred, his compliance to therapy, at times, was limited and may have played a role in control of his disease. Later, in his clinical course, an aggressive anorectal carcinoma developed, appearing to originate from the fistulous tract. Malignant change in fistulous tracts has previously been reported to occur rarely in Crohn's disease[18-24], but development after treatment with biological agents has not been previously recorded.

Malignant complications occur in Crohn's disease. Indeed, several characteristics of colorectal cancer known to complicate the clinical course of Crohn's disease were all confirmed here. These include: duration over a decade, young age at onset of malignancy, and definition of the malignant lesion as a mucinous type carcinoma[12]. This fatal carcinoma could be attributed to the underlying Crohn's disease, possibly related to "adenomatous epithelialization" of the fistula tract[19] with progression from dysplastic epithelium to carcinoma. Neoplastic change could be a consequence of long term exposure to drugs such as metronidazole or azathioprine. However, the potential long-term effects of biological agents in this setting also need to be considered. Recently, advanced colon cancer has been recognized not long after commencement of infliximab therapy[25,26].

Two patients with recent cancer-negative colonoscopies developed advanced and extensive colon cancer within 2 years after initiation of infliximab therapy for their Crohn's colitis[25]. A multicenter study found no increase in the short-term incidence of newly diagnosed neoplasia in infliximab-treated Crohn's. However, the investigators felt that longer term studies with larger patient groups were needed to learn the ultimate effect of these agents[26]. Biological agents could play a role in the initiation of cancers, but in long-standing and extensive Crohn's disease where the risk of neoplasia is already increased, such agents could also affect progression of this process or influence its aggressiveness.

Clinicians who care for Crohn's disease complicated by long-standing and persistently draining fistulas need to be especially wary of the potential for malignancy as a complication. This is especially important for patients most likely to be considered candidates for biological therapy, such as people with persistently draining fistulae that have not responded to other forms of treatment. Modern imaging methods, such as MRI, may be very helpful in detection, especially if biological agents are contemplated for treatment. Published clinical trial data on use of biological agents in Crohn's disease and other disorders are for relatively short term use and do not provide insight into the longer term effects of treatment. The US FDA has appealed to clinicians using approved TNF blockers and caring for these patients to be alert to this issue[27]. While the FDA has required that future studies supply such data, long-term results will not be available for at least another decade. In the meantime, clinicians are encouraged to report suspected or possible adverse reactions or unexpected outcomes during therapy since this may be the only practical means to identify serious problems with such new therapies.

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Meetings

Events Calendar 2010

January 15-16, 2010
AGA Clinical Congress of Gastroenterology and Hepatology
The Venetian And Palazzo, 3355 Las Vegas Blvd South, Las Vegas, United States
http://www.gilearn.org/clinicalcongress

January 16-17, 2010
The Symposium on Clinical Interventional Oncology
Hollywood, Florida, United States

January 22-24, 2010
ASCO Gastrointestinal Cancers Symposium
Orlando, FL, United States

February 05-09, 2010
Cancer Genomics, Epigenomics & the Development of Novel Therapeutics
Waikoloa, HI, United States

February 19-20, 2010
8th International Symposium on the Evolution of Supportive Care in Oncology: the Era of Targeted Agents
New York, NY, United States

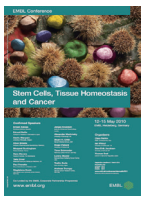
March 04-07, 2010
2010 Annual Meeting of the Society of Surgical Oncology
Renaissance® St. Louis Grand Hotel, 800 Washington Avenue, St. Louis, Missouri 63101, United States
http://www.surgonc.org/

March 05-07, 2010
Genitourinary Cancers Symposium
San Francisco, CA, United States

March 07-11, 2010
16th International Conference on Cancer Nursing
Atlanta, GA, United States

March 25-28, 2010
20th Conference of the Asian Pacific Association for the Study of the Liver
Beijing, China
http://www.apasl2010beijing.org/en/index.aspx

April 17-21, 2010
101st Annual Meeting of the American Association for Cancer Research
Washington, DC, United States



May 12-15, 2010
Stem Cells, Tissue Homeostasis and Cancer
EMBL Heidelberg, Germany
http://www.embl.de/training/courses_conferences/conference/2010/STM10-01/

May 15, 2010
Digestive Disease Week 2010
American Association for the Study of Liver Diseases Ernest N. Morial Convention Center, 900 Convention Center Blvd, New Orleans, LA 70130, United States
http://www.ddw.org/

June 04-06, 2010
American Society of Clinical Oncologists Annual Meeting
Chicago, IL, United States

June 09-12, 2010
13th International Conference on Emergency Medicine
Singapore, Singapore

August 28-31, 2010
10th OESO World Congress on Diseases of the Oesophagus 2010
Boston, Massachusetts, United States

September 23-25, 2010
2010 Gastrointestinal Oncology Conference
The Sheraton Philadelphia City Center, Philadelphia, PA, United States
http://www.isgio.org/isgio2010/program.html

September 23-26, 2010
The 1st World Congress on Controversies in Gastroenterology & Liver Diseases
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October 15-20, 2010
ACG 2010: American College of Gastroenterology Annual Scienitfic Meeting
San Antonio, TX, United States

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Acknowledgments

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1 **Jung EM**, Clevert DA, Schreyer AG, Schmitt S, Rennert J, Kubale R, Feuerbach S, Jung F. Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors: A prospective controlled two-center study. *World J Gastroenterol* 2007; **13**: 6356-6364 [PMID: 18081224 DOI: 10.3748/wjg.13.6356]

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2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

Volume with supplement

7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

No volume or issue

9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer

disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http//www.cdc.gov/ncidod/EID/eid.htm

Patent (list all authors)

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

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