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REVIEW

Effect of exercise on colorectal cancer prevention and treatment

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Abstract

In recent years, because of improved cancer screening, detection and treatment modalities, a rapid increase in the population of colorectal and other cancer survivors has been observed. The increasing population has justified the requirement of preventive strategies such as lifestyle modifications with regard to obesity, physical activity, diet and smoking. Physical activity may prevent approximately 15% of the colon cancers. Furthermore, several observational studies have demonstrated the efficacy and dose-dependent and anti-cancer effects of exercise on decreasing the mortality and risk of recurrence before and after the colorectal cancer (CRC) diagnosis. However, the required exercise dose, type and intensity are yet unclear. The results of randomised prospective studies are expected to determine the optimal amount, type and intensity of exercise and formulate the most appropriate exercise plan and guidelines, according to the requirements and comorbidities of the patients. In addition, recent studies have focused on the molecular and genetic mechanisms underlying the effect of physical activity on disease outcomes and recurrence rates. This review aimed to investigate the effects of physical activity and the biological basis of these effects in preventing the risk and recurrence of CRC and decreasing the hazards of cancer and cancer treatment.

Key words: Colorectal cancer; Exercise; Physical activity

Core tip: This review aimed to investigate the effects of physical activity and the biological basis of these effects in preventing the risk and recurrence of colorectal cancer (CRC) and decreasing the hazards of cancer and cancer treatment. Several observational studies have demonstrated the efficacy and dose-dependent and anti-cancer effects of exercise on decreasing the mortality and risk of recurrence before and after the CRC

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diagnosis. However, the required exercise dose, type and intensity are yet unclear. The results of randomised prospective studies are expected.

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INTRODUCTION

Colorectal cancer (CRC) is the third most common type of cancer and the fourth most common cause of cancer-related death worldwide[1,2]. A significant improvement has been observed in the 5-year survival rates with early screening programs, new treatment modalities and individualised treatments. The 5-year survival rates of patients with stage 1-3 CRC, accounting for approximately 75% of patients with CRC, has approached 65%[3]. Although long-term survival is poor in metastatic disease, patients have been living for more than 2 years because of new developments in treatment strategies[4].

The aetiology of CRC is multifactorial. In addition to genetic factors, lifestyle and environmental risk factors have substantial effects on CRC development. Several risk factors have been identified for CRC (low-fibre and high-fat diet, sedentary lifestyle, diabetes, obesity, smoking, alcohol, advanced age and inflammatory bowel disease). In recent years, the increase in CRC incidence is attributed to the increase in the elderly population, changes in dietary habits and increased risk factors such as smoking, low physical activity and obesity. Despite the advances in treatment strategies, new therapies have limited impact on cure rates and survival. Therefore, a tendency towards selecting adjuvant treatment strategies such as physical activity and exercise has been observed[4-6].

Epidemiological studies have shown that lifestyle factors and obesity affect the development of various types of cancer, particularly CRC[7]. A synergistic association is observed between physical inactivity and obesity. The International Agency for Research on Cancer (IARC) has reported that 25% of all the cancer cases worldwide are caused by obesity and sedentary lifestyle[8].

Obesity and decreased physical activity are associated with colon cancers that have P53 overexpression and KRAS mutation[9,10]. Physical activity may prevent approximately 15% of the colon cancers[8]. An umbrella review, including 19 reviews, 26 meta-analyses and 541 original studies, evaluating physical activity and cancer risk, has shown that regular physical activity is beneficial in preventing 7 types of cancers (colon, breast, endometrium, lung, oesophagus, pancreas and meningioma)[11]. The effect of physical activity on cancer risk is much stronger in breast and colon cancer than in the other types of cancers[12]. In recent studies, a dose-dependent effect of exercise has been reported[13,14].

More than 40% of the patients diagnosed with CRCs have comorbid diseases (diabetes, obesity, chronic obstructive pulmonary disease and heart failure)[15]. Physical activity decreases the risk of developing comorbid diseases in patients with CRC and improves the disease outcomes in patients with comorbid diseases. Several studies have been conducted regarding the effects of exercise on cancer prevention and the outcome in patients with cancer. Most studies focus on CRC and breast cancer. Furthermore, observational studies regarding cancer prevention and exercise are predominant and have often tested aerobic exercise programs. In these studies, physical activities were generally measured as metabolic equivalents hour/week (MET-hour/week). Because these studies include heterogeneous physical activity applications and are survey-based and subjective, significant errors in physical activity measurement have been reported. The studies showed the benefit of increased physical activity after the known predictors (stage, tumour differentiation and treatment status) were adjusted in the analysis. Furthermore, most studies suggested that the beneficial effect of physical activity is independent of the body mass index (BMI) and physical fitness. In recent years, exercise in patients with CRC is used as both primary and secondary prevention and CRC treatment.

CRC AND PRIMARY PREVENTION

Recently, several studies regarding cancer prevention and treatment strategies are directed towards novel approaches alternative to pharmacological treatments. Physical activity has been known to decrease the incidence of age-related chronic diseases such as cardiovascular disease, diabetes, hypertension and metabolic syndrome. Recent studies have additionally revealed that exercise aids the prevention of CRC.

The protective role of physical activity can be correlated with the incidence of precancerous colorectal polyps[16]. In an epidemiological study, it was observed that those who exercised for ≥ 1 h per week had a lower prevalence of colon polyps and adenoma than those who exercised for < 1 h[17]. In this study, exercise decreased the risk of polyp development throughout the entire colon, regardless of a specific area of the colon. In another study, exercise was reported to decrease the total number of intestinal polyps by 50% and the number of large polyps by 67%[18].

A meta-analysis showed that physical activity resulted in a 24% decrease in colon cancer risk [risk ratio (RR): 0.76, 95% confidence interval (CI): 0.72–0.81][19]. In a population of more than 150000 people, 940 colon and 390 rectal cancer cases were detected in a 6-year study period of an epidemiological study, evaluating the association between the risk of colon cancer and physical activity.In this study, 21% of men and 16.5% of women were physically active (> 7 h of regular physical activity), and it was reported that the risk of colon cancer decreased by 40% in the people exercising 7 h a week[20,21].

In the studies evaluating the association between CRC and physical activity, the intensity, type and area-specific effect of physical activity have been evaluated. A meta-analysis showed that increasing doses of physical activity considerably decreased the risk of colon cancer[20].

In a study by Mahmood *et al*[21], no statistically significant decrease in the risk of CRC was detected with occupational, transport and household activities. However, recreational activity significantly decreased the risk of CRC. Although physical activity decreases the risk of colon cancer, the association between physical activity and colon cancer are yet unclear. Furthermore, the duration and intensity of physical activity required to optimally decrease the risk of CRC are unknown.

In recent studies, the epidemiological differences in proximal and distal colon cancers and different genetic and environmental risk factors are reported to show different molecular features. Accordingly, modified treatment approaches have been adopted for proximal and distal colon cancers[22]. In a study, it was reported that physical activity had more effect on the risk of distal colon cancer than that of proximal colon cancers[23].

In a meta-analysis study involving 21 studies, the association between physical activity and colon cancer did not differ with the anatomical location[14]. Moreover, data regarding rectal cancer are insufficient[24]. Some studies describe a similar decrease in the risk of colon cancer, whereas other studies have not demonstrated the benefit of physical activity in rectal cancer[19,25,26]. In a meta-analysis, no significant decrease in risk was found with regard to the incidence of rectal cancer among physically active subjects (RR: 1.15, 95%CI: 0.83–1.64)[27]. In a recent meta-analysis, higher physical activity was found to be associated with a decreased risk of colon by 16% and rectal cancer by 13%[28].

According to some studies, the anti-cancer effect of exercise depends on the carcinogenic exposure and the duration of physical activity. In a preclinical study, exercising during and before chemical exposure in a chemically induced intestinal tumour rat model resulted in a significant decrease in the number of tumours. However, exercise following chemical exposure did not have any effect[29].

In early physical activity and cancer studies, physical activity has been reported to decrease the risk of colon cancer, particularly in men; however, it does not decrease the risk in women[30]. Mechanisms associated with gender differences are yet unknown. Sex hormones are considered to have a protective effect against colon cancer; thus, the difference observed in the studies may be because of a decreased protective effect caused by decreased estrogen levels in women who exercise more. However, in the meta-analysis of 20 studies in 2011, no difference was observed between the genders in terms of physical activity and the risk of CRC[31]. Moreover, in a study involving postmenopausal women, women undergoing hormone replacement therapy were shown to have a lower risk of colon cancer, and physical activity did not provide any additional benefit. However, it has been shown that physical activity has a protective effect against colon cancer in postmenopausal women who are not undergoing hormone replacement therapy[32]. In a meta-analysis evaluating 19 cohort studies, the risk of CRC in physically active women and men was reported to be decreased by 29%and 22%, respectively[33].

CRC AND SECONDARY PREVENTION

Because of early diagnosis and advances in the treatment of cancers, > 25 million people have been diagnosed with cancer worldwide, and this number is increasing each day. This population has been estimated to increase to > 75 million in the next 3 decades[34]. Despite positive developments, patients experience several long-term health issues and physical and psychological issues following cancer treatment. Compared with the general population, studies have shown that secondary malignancy, cardiovascular disease, diabetes, osteoporosis, sleep disorders, anxiety, depression and decreased functional capacity exhibit a higher risk, in addition to the risk of recurrence, in patients with cancer. These comorbid conditions can be caused by cancer treatment, in addition to genetic predisposing and lifestyle factors[35,36]. In recent years, there has been an increasing awareness of lifestyle modifications because of the increased population of cancer survivors.

With regard to primary prevention, the role of physical activity during and after CRC treatment is yet unclear. Compared with other cancer survivors, CRC survivors have been observed to have a higher rate of physical inactivity[37]. In one study, 68% of CRC survivors were physically inactive after a curative treatment[38]. Despite the benefits of physical activity, only 23.5% of CRC survivors followed the exercise guidelines[39]. In another study, 21%–42% of CRC survivors could exercise according to the guidelines recommended by the American College of Sports Medicine (ACSM)/American Cancer Society (ACS)/National Comprehensive Cancer Network, 6 months following the curative treatment[40]. Adherence to exercise and dietary guidelines in patients with cancer is associated with a lower incidence ofcancer and lower cancer-specific and all-cause mortality[41].

Despite advances in the diagnosis and treatment, the rate of recurrence for locally advanced colon cancer is 40%[42]. In addition to the risk of recurrence, CRC survivors experience the late and long-term effects of cancer treatment[43,44]. Lifestyle interventions such as an improved diet and exercise are recommended to improve the side effects of cancer and cancer treatment. Physical activity improves clinical conditions, such as weakness, quality of life, muscle strength, lymphedema, depression, functional status, and decreases the risk of recurrence of cancer before and after the diagnosis of CRC and cancer-specific and overall mortality[38,45-47]. Because exercise decreases the risk of developing chronic diseases such as cardiovascular disease and diabetes, there is a decrease in the all-cause mortality[48].

In a meta-analysis evaluating 16 breast and 7 CRC studies comparing low and high levels of post-diagnostic physical activity, a 42% (RR: 0.58, 95%CI: 0.48–0.70) decreasein the risk of all-cause mortality and 39% decrease in CRC-specific mortality (RR 0.61, 95%CI: 0.40–0.92) were detected[49]. In CRC survivors who adhered to the new guidelines, CRC-specific mortality has been shown to be 10%–40% less and all-cause mortality to be 20%–50% less[50-53]. Clearly, post-diagnostic physical activity is associated with an improvement in disease outcomes in patients with CRC; however, whether the pre-diagnostic physical activity affects the CRC survival remains unclear[54]. A meta-analysis evaluating prospective cohort studies has shown a 25% decrease (HR: 0.75, 95%CI: 0.65–0.87) in CRC-specific mortality in those who participated in any physical activity at any level before diagnosis compared with those who did not participate at all. Furthermore, the pre-diagnostic physical activity decreased the all-cause mortality[55]. The clinical benefit rate increased when the level of physical activity was increased. Whether the effect of the post-diagnostic physical activity on CRC survival is influenced by the pre-diagnostic physical activity remains unknown. A new meta-analysis included both pre-diagnostic (CRC-specific mortality; HR: 0.79, 95%CI: 0.71–0.89, total mortality HR: 0.81, 95%CI: 0.72–0.91) and post-diagnostic physical activity (CRC-specific mortality; HR: 0.77, 95%CI: 0.63–0.94, all mortality HR: 0.71, 95%CI: 0.63–0.81)and confirmed its association with improved disease outcomes[56]. Furthermore, a recent study demonstrated the benefit of both pre- and post-diagnostic activity in postmenopausal women with CRC[57].

CRC is associated with multiple gene mutations (such as *APC, KRAS, PIK3CA* and *TP53*). Recent studies to identify patients with CRC who may benefit from exercise evaluated the association between physical activity and cancer outcomes using molecular [KRAS, PIK3CA, BRAF and microsatellite instability (MSI)] or genetic markers [P27 (CDKN1B)-positive, B-catenin (CTNNB1)-negative, PTGS2 (prostaglandin-endoperoxide synthase 2/COX-2)-positive or the insulin receptor substrate (IRS1)-low/negative protein expressions][58-62]. The study results showed that P27, B-catenin, COX-2 and IRS1 expression significantly modified the association between the post-diagnostic physical activity and CRC-specific survival. Physical activity significantly has been shown to improve CRC-specific survival after diagnosis in patients with tumours with increased P27 and COX-2 expression and decreased B-catenin and IRS1 expression[58-62]. Hardikar *et al*[63] showed that the beneficial effect of

physical activity was not specific to the molecular phenotype of CRC (*BRAF* mutation, *KRAS* mutation and MSI status).

To date, few studies have evaluated the feasibility and reliability of increased physical activity compared with the standard levels. In a study by Brown *et al*[5], stage 1–3 colon cancer survivors were included in anaerobic exercise program for 150 min/wk (14 patients, low doses) or 300 min/wk (12 patients, high doses) for 6 months, and 13 patients were randomised into the control group. In this study, changes in the prognostic markers such as soluble intercellular adhesion molecule (sICAM-1) and vascular adhesion molecule 1 (sVCAM-1), which are associated with early death and disease recurrence, were evaluated among colon cancer survivors[64,65]. Increased exercise and physical activity (300 min/wk) were associated with decreased mortality and risk of recurrence in CRC survivors. Furthermore, sICAM-1 reduction was achieved in both exercise arms; however, sVCAM-1 did not decrease. Moreover, sICAM-1 may be associated with the anti-cancer effect of exercise; however, this finding requires further confirmation[66].

Although observational studies have shown the beneficial association between physical activity and survival after CRC treatment, no randomised controlled trials have been conducted. The first prospective phase 3 randomised clinical trial, Challenge Trial (the Colon Health and Lifelong Exercise Trials), continues to evaluate the effect of 3-year exercise on survival in stage 2 and 3 CRC survivors[67]. Furthermore, there are many on-going studies focusing on CRC and exercise (Table 1).

EXERCISE IN PATIENTS WITH CRC UNDERGOING TREATMENT

Reportedly, exercise improves the surgical tolerance of CRC and decreases the hospital stay after surgery[68]. Exercise before CRC surgery may improve postoperative results. However, one review concluded that whether exercise before CRC surgery reflected improvement in peri- and post-operative outcomes was unclear[69]. A phase 3 randomised prospective study, PHYSSURG-C study, evaluating the effect of pre-and post-operative physical activity on the post-operative morbidity and mortality after CRC surgery is on-going (NCT 02299596)[70]. Exercise has been shown to improve the quality of life and decrease few side effects in several patient groups receiving adjuvant therapy[44]. In addition, exercise increases the completion rate of chemotherapy in patients with CRC[71].

Neoadjuvant chemotherapy and radiotherapy can cause severe acute toxicity in locally advanced rectal cancer. Few studies have shown that exercise is feasible and safe during neoadjuvant therapy in rectal cancer[72]. The EXERT study, which is evaluating the effect of exercise on the clinical outcomes and side effects of exercise performed during and after neoadjuvant treatment in locally advanced rectal cancer, is on-going (NCT03082495)[73].

In patients with cancer, the disease itself and each treatment modality applied (surgery, chemotherapy and radiotherapy) can create specific side effects and complications that affect their daily life. Side effects such as fatigue, pain, muscle weakness, peripheral neuropathy, cardiovascular and pulmonary complications, endocrine changes, anaemia, immune dysfunction, sleep disorders, depression, anxiety, gastrointestinal disturbance and skin changes can develop during the treatment. The most common side effects during the treatment in patients with CRC are generalised and muscle weakness. In these patients, physical exercise programs improve the symptoms and side effects of chemotherapy, along with the quality of life[5,74,75]. In the Cochrane study, which included 56 randomised trials involving patients with cancer-associated fatigue, exercise was found to decrease cancer-associated fatigue and improve depression and sleep disorders[76]. The 6- and 12-wk home-based exercise programs, which are easier to apply, have been shown to significantly improve physical fitness in CRC survivors and to be effective and applicable in increasing the level of physical activity[77,78]. The CASUS (Cancer Survivor Study), a prospective observational study investigating the effect of physical activity and nutrition on the quality of life and disease recurrence and survival in CRC survivors, is on-going[79].

Patients with metastatic disease have very low participation rates in exercise programs. Prospective studies investigating the effect of exercise on clinical outcomes have shown that physical activity improves prognosis in patients with CRC even in advanced stage patients[52]. In a study regarding patients with stage 4 CRC, an improvement in fatigue, functional capacity, sleep quality and the quality of life was reported in patients after 8 weeks of home-based exercise programs[80]. Moreover, low

Table 1 Ongoing trials on colorectal cancer and exercise

Study	Conditions	Interventions	Title	Status
NCT01325909	Rectal cancer	Exercise programme	Exercise training in colorectal cancer patients	Completed
NCT03515356	Colorectal cancer	Motivational interviewing-walk intervention/physical activity education pamphlet	Exercise to reduce chemotherapy-ınduced peripheral neuropathy	Recruiting
NCT01924897	Colorectal cancer	Exercise training	Preop cardiopulmonary exercise testing and exercise training in colorectal patients	Unknown
NCT00985400	Colorectal cancer	Exercise programme/telephone-based intervention	Doctor-recommended home-based exercise program or relaxation training in improving physical function and controlling symptoms in patients with stage IV or recurrent colon cancer that cannot be removed by surgery	Active,not recruiting
NCT00230646	Colon cancer/rectal cancer	Exercise counseling	Promoting physical activity after colorectal cancer	Completed
NCT01133132	Colon cancer	Survivorship CHESS (mobile comprehensive health enhancement support system)	Interactive cancer communication system directed physical activity enhancement for colon cancer survivors	Completed
NCT02597075	Metastatic colorectal cancer	Standard therapy+physical activity program/standard therapy	Physical activity in patients with metastatic colorectal cancer who receive palliative first line chemotherapy	Recruiting
NCT02191969	Colorectal Cancer/fatigue	Walk with ease	Physical activity ıntervention for older patients during chemotherapy for colorectal cancer	Recruiting
NCT02966054	Colon cancer/rectal cancer	Digital health physical activity ıntervention group	Self-monitoring and reminder texts to ıncrease physical activity after cancer:a pilot randomized controlled trial	Completed
NCT00373022	Colorectal Cancer/depression/anxiety disorder	Exercise programme	Moderate physical activity in helping patients recover physically and emotionally from stage II or stage III colorectal cancer	Completed
NCT01708824	Colorectal cancer	Physical activity/dietary	Diet and physical activity intervention in CRC survivors	Unknown
NCT03232814	Colorectal cancer	Group-based walking	Walk on:a community-based approach to ıncrease physical activity among men treated for colorectal cancer	Withdrawn
NCT01991847	Colorectal cancer	Physical activity	Tertiary prevention by exercise in colorectal cancer therapy	Completed
NCT02056691	Colorectal cancer	Exercise programme/muscle biopsy	Exercise induced changes in colorectal cancer tissues	Completed
NCT00819208	Colorectal cancer/anxiety/depression/fatigue/sleep disorders	Exercise intervention	Health education materials with or without a physical activity program for patients who have undergone treatment for high risk stage II or stage III colon cancer	Recruiting
NCT02347852	Colorectal neoplasms	Regorafenib	Assessment of physical activity during therapy with regorafenib for metastatic colorectal cancer	Completed
NCT02780284	Colorectal cancer	Physical activity intervention	Microbiome, exercise tracking study	Unknown
NCT02250053	Colon cancer (stage 2 and 3)	Exercise	Exercise and colon cancer	Unknown

NCT03049124	Rectal cancer	Exercise	Exercise for adults diagnosed with rectal cancer	Recruiting
NCT03082495	Rectal cancer	Exercise	Exercise during and after neoadjuvant rectal cancer treatment	Recruiting
NCT03111823	Stage IV colorectal cancer	Exercise intervention	Exercise program during chemotherapy in metastatic colorectal cancer	Terminated
NCT00977613	Colorectal cancer	Exercise counseling	Adherence to a recommended exercise regimen in colorectal cancer patients	Completed
NCT03291951	Colon cancer	Resistance training	Focus on reducing dose-limiting toxicities in colon cancer with resistance exercise study	Enrolling
NCT02264496	Colorectal cancer	Exercise	Prospective randomised trial of exercise and/ prantioxidants in colorectal cancer patients undergoing surgery	Completed
NCT03120104	Rectal cancer	Pelvic floor muscle exercise	Physical exercise for colorectal cancer patients after transanal total mesorectal excision	Recruiting
NCT03186638	Colorectal cancer (stage 1-3)	Exercise intervention/ibuprofen	Exercise and low-dose ibuprofen for cognitive ımpairment in colorectal cancer patients receiving chemotheapy	Recruiting
NCT00668161	Colon cancer prevention	Exercise	Effect of exercise on biomarkers of colon cancer risk	Completed
NCT02538913	Rectal neoplasms	Exercise training	Exercise training for rectal cancer patients	Recruiting
NCT02724306	Colon polyps/adenomas	Active lifestyle programme	Physical activity intervention with people at ıncreased risk of developing colon cancer	Completed
NCT01859442	Locally advanced rectal cancer	Structured responsive interval exercise training programme	The effects of cancer therapies and exercise on mitochondrial energetics and fitness	Completed
NCT01914068	Rectal cancer	Supervised exercise in hospital	The effects of a 9 wk exercise programme on fitness and quality of life in rectal cancer patients after chemoradiotherapy and before surgery	Completed
NCT02057991	Colorectal cancer/anxiety/depression/ fatigue	Educational intervention/CAM exercise therapy (mindfulness exercise video)	Mindfulness-based exercise video in educating Hispanic/Latino patients with colorectal cancer and their caregivers	Terminated
NCT02403024	Colorectal cancer	Interval walking	Feasibility and Efficacy of Interval Walking in patients with colorectal cancer	Completed
NCT02188342	Colorectal cancer	High intensity interval training	Assessing the effectiveness of a preoperative high intensity interval training programme in older colorectal cancer patients	Completed
NCT03336229	Colorectal cancer	Exercise Intervention	Enhancing fitness with preoperative exercise in colorectal cancer surgery	Not yet recruiting
NCT02586701	Colorectal cancer	Supervised /non-supervised exercise	Supervised versus non-supervised exercise on adherence and functional outcomes in colorectal patients	Completed

NCT01210313	Colorectal cancer	Physical activity	Physical activity for reduction of recurrence rate after adjuvant chemotherapy for localised colorectal carcinoma	Completed
NCT02299596	Colorectal cancer	Physical activity	Physical activity in relation to surgical procedures	Recruiting
NCT03361150	Colorectal cancer	Physical activity	High-intensity interval *vs* moderate continuous training in surgical prehabilitation	Completed
NCT02889276	Colorectal cancer	Unsupervised activity/Functional resistance training	Effects of functional exercise on fitness and QoL in cancer survivors	Recruiting
NCT02895464	Colorectal cancer	Exercise	Feasibility of home-based preoperative exercise in older people	Completed
NCT02499939	Colorectal cancer	Exercise/ultrasound therapy	Ultrasound therapy and therapeutic exercise for chemotherapy ınduced peripheral neuropathy	Completed
NCT02522520	Colorectal cancer	Pedometer intervention	Pedometer Intervention and health effects for sedentary colorectal cancer patients during adjuvant chemotherapy	Recruiting
NCT02442583	Colorectal cancer	Brochure regarding sedentary behavior	Reducing sedentary behaviors among colorectal cancer survivors	Completed

intensity physical activities in patients with stage 4 metastatic cancer are recommended. The studies have shown no clear association between the physical activity and overall or CRC-specific survival in patients with metastatic CRC[81,82].

The CALGB89803/ALLIANCE study, a prospective observational study, investigated the effect of physical activity on survival in patients with colon cancer before and after recurrence 6 months post-completion of adjuvant therapy. After adjusting the potential factors that can affect the survival, a statistically significant 29% improvement in mortality was observed in physically active patients with recurrent colon cancer[83]. The physical activity prior to recurrence appears to be a factor that affects prognosis in patients with recurrent colon cancer.

OBSTACLES, CONTRAINDICATIONS AND COMPLICATIONS FOR EXERCISE

Although it is known that exercise has beneficial effects in patients with CRC and survivors, there are some obstacles to participation of patients in exercise regimens. Fatigue is the most frequently reported obstacle to exercise in patients with CRC[84]. More than 60% of patients with cancer complain of weakness during and after treatment. Fatigue decreases the quality of life and physical activity. The cause of fatigue in patients with cancer is unknown, and may develop because of the disease itself or its treatment. Furthermore, it may occur because of other clinical issues such as depression, physical inactivity and sleep disorders. Although complaints such as nausea and pain can be treated effectively during cancer treatment, effective treatment for fatigue is yet unavailable. After treatment, fatigue decreases over time; however, 30% of the patients may continue to complain for years[85]. In addition, treatment-related side effects and comorbid conditions (cardiopulmonary disease and diabetes) may be an obstacle to exercise. CRC survivors with peripheral neuropathy have been shown to participate in lesser physical activity than those without neuropathy[86].

In addition to the effect of exercise on survival, several studies have evaluated the efficacy and reliability of physical activity applications (home-based, supervised and telephone-based counselling and interval walking) in CRC survivors. These studies have shown that aerobic and resistance exercises were safe and did not increase the risk of side effects throughout the chemotherapy[80,87-89].

Understanding the factors that impede exercise practices is important for directing patients with cancer to the appropriate exercise programs. However, data regarding the appropriate exercise dose and type to safely correct the results is insufficient. Guidelines should be developed according to each patient's disease, age and

comorbid condition. Clinicians can influence patients by encouraging them to exercise, thereby increasing patient participation in physical activity. However, in a study investigating the effect of oncologists' exercise advice on patients, oncologists' exercise re-commendations alone were shown to be insufficient to increase participation in exercise. Reportedly, exercise package programs may be ideal for increasing the exercise participation[90].

Contraindications for exercise are heart failure, acute infectious disease, metabolic disease (thyrotoxicosis and myxoedema) and mental and physical disorders[91]. Non-scheduled exercises can cause complications. Furthermore, intense physical activity, particularly in the early period, can result in deterioration of wound healing and parastomal hernias. Skeleton stability should be investigated before exercise, if patients are suspected of bone metastasis. Blood counts should be monitored in patients who are scheduled to participate in exercise during chemotherapy. For intensive and light exercises, thrombocytes should be at least 50000/μL and 20000–50000/μL, respectively. If haemoglobin is ≤ 8 mg/dL, exercise may cause cardiac ischemic complications because of increased O_2 requirement. Neutropenia is not a contraindicated condition for exercise; however, caution should be exercised in terms of infection. Patients should avoid severe exercises because of the toxicity (nausea, vomiting, nephrotoxicity, cardiotoxicity and diarrhea) caused within 24 h of chemotherapy[24]. Severe peripheral neuropathies constitute the contraindications for exercises. Physical activity studies have reported no side effects because of exercise during and after cancer treatment[91].

EXERCISE AND MECHANISMS OF ACTION

Although the association between exercise and prevention of CRC is definite, the molecular mechanism underlying the protective effect of exercise is yet unknown. The association between exercise and cancer is explained through several mechanisms. These mechanisms include metabolic dysregulation [involving insulin, glucose and insulin-like growth factor (IGF)], sex hormones, adiposity [changes in adipokines (leptin andadiponectin)], oxidative stress and inflammation and impaired immune function[42,92] (Figure 1).

Insulin pathway

Insulin influences DNA synthesis, cell survival, proliferation and differentiation using various cellular signalling pathways via insulin growth factor receptor IGF1R[93]. Elevated systemic IGF1 levels are associated with CRC risk[94]. In preclinical studies, exposure to insulin has been shown to induce colonic tumour cell development[95-97]. Moreover, observational studies have supported pre-clinical studies[98,99]. Insulin resistance is associated with higher CRC incidence and mortality[100]. In the studies, the degree of IGF1R over-expression has been shown to be correlated with the tumour stage in CRCs[101].

Physical activity decreases insulin resistance and the insulin levels affecting the IGF pathway and indirectly decreases the risk of CRC, recurrence and mortality. With regard to the IGF pathway, heterogeneity is observed in response to exercise. In some studies, an increase in IGF-1 and IGFBP-3 levels was observed with exercise, whereas in some cases, a decrease was observed. This clinical condition may be because of several factors affecting the association between the IGF pathway and exercise[102]. Despite heterogeneous results, decreased IGF-1 levels and increased IGFBP-3 levels may be a reasonable mechanism underlying the inverse correlation between CRC and physical activity[103]. The association between exercise and CRC cannot be explained using a single mechanism because exercise and interrelated factors exert varying effects.

Inflammation

Inflammation is known to be a risk factor for various chronic diseases (obesity and metabolic syndrome) including cancer. Inflammation plays an important role in cancer development and progression[104]. Although the underlying mechanisms are yet unclear, the inflammatory process appears to be an important pathway associated with the risk of CRC. Physical activity can decrease systemic inflammation and improve immune function[105]. Proinflammatory cytokines such as IL-6, C-reactive protein and tumour necrosis factor (TNF)-α are associated with an increased risk of cancer. Various studies have demonstrated the effects of physical activity on IL-6 in the colon cancer model. In a study conducted by Mehl *et al*[106], a decrease in plasma IL-6 was observed in $APC^{min/+}$ male mice after treadmill running. This result has been shown to be associated with fewer polyps.

New preclinical studies have shown that inflammation is associated with polyp

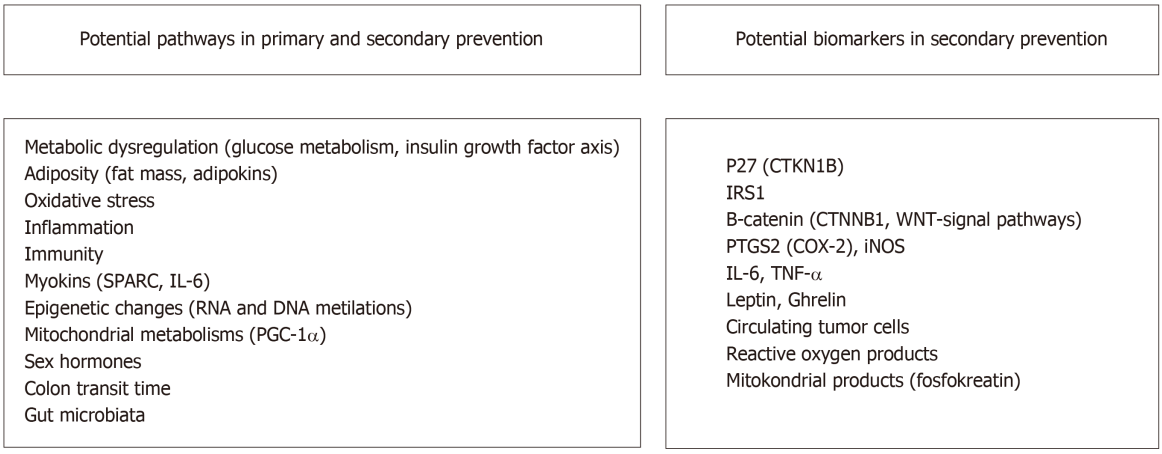

Figure 1 Biological potential pathways as explanatory mechanisms of the association between physical activity and primary and secondary prevention of colorectal cancer/potential biomarkers in secondary prevention. SPARC: Secretory protein acidic and rich in cysteine; PGC-1 α: Peroxisome proliferator-activated receptor gamma co-activator 1α; CTKN1B: Cyclin-dependent kinase inhibitor 1B; IRS1: İnsulin receptor substrate 1; CTNNB1: Catenin beta 1; PTGS2: Prostaglandin-endoperoxide synthase 2; COX-2: Cyclooxygenase-2; TNF- α: Tumor necrosıs factor alpha; iNOS: İnducible nitric oxide synthase.

formation and progression and that the cyclooxygenase isoenzymes (COX-1 and -2) particularly play an important role in intestinal tumour formation[103]. Administration of nonsteroidal anti-inflammatory drugs that inhibit the COX enzyme is known to be associated with a decreased risk of colon cancer (RR: 0.60, 95%CI: 0.40–0.89)[107,108]. Physical activity results in a local anti-inflammatory effect by decreasing COX-2 and iNOS (inducible nitric oxide synthase) expression in the colon mucosa. Adipocyte, energy balance, insulin, adipokines, estrogen and other factors known to play a role in carcinogenesis have been shown to affect the inflammatory response. Therefore, these factors may be involved in the indirect effect of physical activity on inflammatory processes in cancer[108,109].

Myokines

Myokine secretion from the skeletal muscles may be involved in the protective effect of exercise. Studies have shown that exercise-induced myokines include IL-6, IL-8, IL-15, brain neurotrophic factor and leukaemia inhibitory factor released from the muscle fibres. Exercise enhances the insulin sensitivity through these cytokines and decreases the production of proinflammatory cytokines[110-112].

Recent studies have shown that the secretory protein acidic and rich in cysteine (SPARC protein), a cellular matrix protein known as a myokine, is released from the muscle tissue after exercise and is involved in intercellular interaction and cell differentiation. The SPARC protein forms a biological association between colon tumorigenesis and physical activity. The SPARC protein prevents CRC development by increasing apoptosis[113]. In a study conducted by Aoi *et al*[114], the antiproliferative and proapoptotic effects of the SPARC protein in colon cancer cells has been shown.

Immunity

The mechanisms underlying the protective effect of exercise on the risk of colon cancer are complex. The role of exercise in the immune system in cancer prevention is yet unclear. Recently, macrophages and T cells have been the important factors in colon cancer pathogenesis. Accumulation of intra-tumoral macrophages is associated with poor prognosis in colon cancer[115,116]. Exercise-induced changes in the immune system are a possible mechanism. In a preclinical study, exercise was shown to affect the immune cell parameters in the mucosal tissue of $APC^{min/+}$ mice. Exercise decreased the expression of macrophage and regulatory T-cell markers and increased the number of cytotoxic T-cells[117]. Other preclinical studies confirmed that exercise was particularly influenced immune cells such as T lymphocytes and macrophages[118,119]. Furthermore, exercise may have a positive effect on immune aging[120]. Exercise has been shown to increase natural killer cell cytotoxicity, monocyte and macrophage number and function and the CD8 T-cell ratio. Furthermore, it has been shown to decrease the increased antigen presentation, inflammation and number of proinflammatory monocytes and prevent the accumulation of aged T-cells[121-123]. These mechanisms demonstrate the complexity of interaction between the risk of cancer and physical activity. Further studies are required to fully understand the associations among immunity, exercise and cancer.

Other mechanisms

In CRC, there are other suggested mechanisms (micro RNA, global DNA methylation, intestinal microbiota, colon transit time and mitochondrial dysfunction) underlying the effects of exercise on tumorigenesis. In recent years, it has been suggested that intestinal microbiota is associated with CRC incidence and progression and may predict the response to immunotherapy. Diet and lifestyle changes alter the intestinal microbiota[124,125]. Several studies have shown that some gut microbes such as anaerobic bacteria significantly increased in patients with CRC; however, further investigation is required to assess the importance of these bacteria and their metabolites in CRC pathogenesis. Moreover, the effect of lifestyle on the anticancer immune response is yet unclear[126].

Another mechanism that explains the association between exercise and CRC is that the exercise decreases the colon transit time. Thus, the interaction of intra-colonic chemicals with colonic mucosa is limited[127]. Moreover, CRC development and risk factors such as obesity and aging are associated with mitochondrial dysfunction. In a recent study, it was shown that the peroxisome proliferator-activated receptor gamma co-activator 1α (PGC-1α), the major regulator of mitochondrial functions, may be a biomarker involved in the protective effect of physical activity in patients with CRC[128].

BIOMARKERS AND THE EFFECT OF EXERCISE ON PATIENTS WITH CRC

Physical activity before and after the diagnosis in CRC is associated with improved disease outcomes and decreased risk of recurrence; however, the underlying molecular mechanisms are unknown. Various observational studies have focused on whether the different molecular properties of CRC affect the association between physical activity and survival. As observed in the standard oncology treatments, every treatment does not have the same effect on every patient. Similarly, physical activity should not be expected to benefit all patients. The molecular basis of the association between CRC and physical activity must be highlighted, and tumour biomarkers or patient characteristics that can predict the response to exercise must be identified. Using molecular markers and protein expression, a patient subgroup that can benefit the most from physical activity can be determined (Figure 1).

P27

P27 loss is common in CRC. P27 is a cyclin-dependent kinase inhibitor that is additionally associated with the insulin pathway. High insulin and IGF-1 levels result in P27 downregulation. In response to energy restriction and physical activity, P27 expression increases[59]. High P27 (CTKN1B) levels are associated with cell cycle termination[129]. The benefit of physical activity may be affected by tumour P27 status. In a preclinical study, patients with colorectal tumours that expressed P27 experienced greater benefit from physical activity after diagnosis than those with a P27 loss. People with ≥ 18 met h/week of physical activity after diagnosis showed a 67% decrease in colon cancer-specific mortality compared with less active subjects. No statistically significant association was observed between the patients with tumour and loss of P27[59].

B-catenin

The B-catenin (WNT) signalling pathway plays an important role in CRC development, energy metabolism, adipogenesis, obesity and metabolic diseases. The activation of the WNT signalling pathway because of a loss of APC and its major mediator CTNNB1 (B-catenin) results in cell growth independent of the energy balance[130]. Physical activity alters the WNT-CTNNB1 signal in the mouse colonic mucosa and the WNT-CTNNB1 signalling pathway affects the cellular sensitivity to physical activity[131].

In one study, patients with early-stage CRC with CTNNB1-negative tumours, who had ≥ 18 met hour/week of physical activity after diagnosis, showed a 67% decrease in the risk of CRC-specific mortality compared with that in inactive patients. However, no correlation was observed in CTNNB1-positive patients[60]. In a study evaluating whether B-catenin predicted the benefit of exercise in patients with metastatic colon cancer receiving chemotherapy, it was found that exercise did not affect the survival (HR: 0.98, 95%CI: 0.32–2.97). However, patients with weak staining for B-catenin in the exercise program had a lower mortality rate (HR: 0.39, 95%CI: 0.025–6.1)[132]. In clinical practice, CTNNB1 status can be used as a predictive biomarker in response to exercise applications[133].

PTGS2 (COX-2) and TNF-α

Physical activity can affect inflammation-induced cell growth. In preclinical studies, exercise has been shown to alter chemically induced COX-2 (cyclooxygenase-2) expression and cell proliferation in the colon. CRC-specific survival may vary based on the PTGS2 (prostaglandin-endoperoxide synthase 2/COX-2) expression status[134]. Among 382 patients with PTGS2 (COX-2)-positive CRC, those with the highest physical activity had an 82% decreased CRC-specific mortality compared with the least active patients. However, the protective effect was not observed in 223 patients with PTGS2-negative CRC[61]. High TNF-α expression in colon tumour tissue is associated with positive lymph node stage and colon cancer recurrence[135]. In patients with colon cancer, the circulating TNF-alpha levels were observed to be decreased by exercise[88].

Insulin, IGF-1 and IRS1

Diabetic patients and patients with metabolic syndrome have an increased risk of CRC recurrence[136]. In addition, increased insulin and IGF-1 levels in patients with CRC are associated with a poor prognosis[137]. In one study, postoperative physical activity in CRC survivors decreased insulin levels and insulin resistance and increased IGF-1 (17.8%, *P*: 0.007) and IGFBP-3 (30.3%, *P*: 00013) levels[88]. However, the decrease in insulin and IGF levels is known to decrease the risk of CRC and improve survival outcomes[104].

İnsulin receptor substrate 1 (IRS1), insulin and IGF are mediators in the insulin signalling pathway, and downregulation of IRS1 is associated with insulin resistance[138]. In a study evaluating 371 patients with stage 1–3 CRC, post-diagnostic physical activity significantly improved the CRC-specific survival in patients with low IRS1-expressing tumours,with a hazard ratio (HR) of 0.15 (95%CI: 0.02–1.38) in the IRS1-negative group, 0.45 (95%CI:0.19–1.03) in IRS1-low group and 1.32 (95%CI: 0.50–3.53) in IRS1-high group.If confirmed by other studies, it may be used as a predictive marker to identify the patient groups that will benefit the most from exercise[62].

Leptin and Ghrelin

Preclinical studies have shown that exposure of colon cancer cells to adipocytes and pre-adipocytes increases cell proliferation[139]. Exercise decreases visceral obesity. Physical activity and exercise decrease inflammatory adipocytes and increase anti-inflammatory adipokines[140]. Leptin and ghrelin are important regulating hormones in the intake and consumption of energy and weight control. Decreased body fat mass and percentage is associated with an increase in ghrelin levels[141]. Ghrelin was found to be associated with increased proliferation and invasion in CRC by endogenous and autocrine effects[142]. The endocrine and autocrine effects of ghrelin in CRC vary. Aerobic exercise causes a decrease in leptin and an increase in ghrelin[143,144]. In one study, because of the increase in adipose tissue, a decrease in ghrelin levels and an increase in the risk of colon cancer were determined[145]. In a study evaluating the effect of 8 weeks of exercise on plasma leptin and ghrelin levels in patients with CRC, a significant increase in ghrelin levels was observed in the exercise group after 8 wk. Plasma leptin levels and insulin resistance didnot differ significantly. Plasma ghrelin levels were negatively correlated with the body fat percentage. If confirmed by other studies, ghrelin hormones can be used as biomarkers to demonstrate the benefit of exercise.

Other biomarkers

Genetic and epigenetic changes induced by reactive oxygen products may contribute to CRC progression. Chronic exercise may decrease the risk of recurrence by decreasing the systemic oxidative stress[146,147].

Circulating tumour cells predict stage 1–3 colon cancer recurrence and mortality[148]. In a study, patients with stage 1–3 colon cancer were randomised into two groups and were subjected to aerobic exercise for 150 min/wk and aerobic exercise for 300 min/wk for 6 mo. After 6 mo, a significant decrease was observed in tumour cells circulating in both low- and high-dose exercise arms. However, no exercise dose-response association was observed[149]. The mechanism underlying the effect of exercise against the circulating tumour cells is yet unknown.

In a recent study, it was observed that the central carbon metabolism was affected, and a significant decrease in phosphocreatine levels was observed in the tumour models that responded to exercise. This finding indicates a change in tumour energy metabolism after exercise in CRC. Furthermore, this study showed that the underlying mechanism of exercise benefit may be the modifications in tumour cell mitochondrial metabolism[150]. Aerobic exercise has been shown to be effective through various intra-tumoral and systemic mechanisms in cancer onset, progression

and metastasis. Furthermore, exercise can activate different biological mechanisms at different intensities[151].

EXERCISE AND GUIDELINES

The ACS and ACSM have developed physical activity and dietary guidelines for patients with cancer. To recommend specific exercise programs, the patient's comorbid conditions and exercise contraindications should be carefully evaluated through medical screening. In addition, the patient's age, gender, type of cancer treatment and physical performance should be considered. Echocardiography should be performed in patients who have a history of cardiac disease or a cardiotoxic chemotherapy regimen. Patients with a history of severe smoking and suspected pulmonary dysfunction should undergo a respiratory function test[24]. If no contraindications (presence of widespread lytic bone metastases, severe thrombocytopenia, anaemia, fever or presence of active infection or safety issues) are present, it is advisable to offer moderate personalised aerobic exercise programs for most patients. Exercise programs vary according to the type, intensity and frequency of exercise[152]. For colon cancer survivors, supervised exercise is an appropriate program[153]. In patients with advanced cancer, the applicability of exercise programs is limited.

Initial intensity and duration of the exercise should be determined according to the functional capacity and comorbid status of the patient. Patients should be initially subjected to exercise with less intensity and duration; furthermore, it should be gradually increased based on patients' medical conditions. For adult patients with cancer who have fatigue, the American Society of Clinical Oncology manual recommends 150 min/wk of moderate aerobic exercise (fast walking, cycling or stretching) and two or three power-boosting exercises (weight lifting)[154].

Recommendation of the 2012 ACS guideline: It is recommended to avoid inactivity and to return to normal daily activity immediately after diagnosis, aiming for at least 150 min/wk of moderate or 75 min/wk of vigorous aerobic exercise, including exercises that require strength at least 2 d/wk[36]. However, it is recommended that this amount must be increased to 225 min (45 min/d, 5 d/wk) in appropriate patients. All patients with cancer should be encouraged to participate in regular physical activity during their lifetime.

CONCLUSION

In recent years, various studies have been conducted regarding the effect of exercise on cancer prevention and clinical outcomes. Increasing observational and experimental evidence suggests that exercise can modify the biology of CRC. Based on the results of the study, lifestyle interventions that provide an improvement in diet and exercise are recommended as an effective method to prevent CRC and improve the negative effects of cancer and its treatment. However, the required exercise dose, type and intensity are yet unclear. The results of randomised prospective studies are expected to determine the optimal amount of exercise, type and intensity and develop the most appropriate exercise plan according to the requirements and comorbidities of the patients and eventually formulate more useful guidelines. In this review, we outlined the beneficial effects of exercise in the prevention and treatment of CRC and the potential biological mechanisms underlying these beneficial effects.

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MINIREVIEWS

Stereotactic body radiation therapy in patients with hepatocellular carcinoma: A mini-review

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Abstract

Stereotactic body radiation therapy (SBRT) is an emerging treatment for hepatocellular carcinoma. This technique results in excellent local control rates with favorable toxicity profile despite being predominantly used in heavily pretreated patients or those unsuitable for other local therapies. SBRT may be used as a sole treatment or in combination with other local therapies as well as a bridging strategy for patient awaiting liver transplants. This brief review describes current practice of SBRT with respect to radiation technique, patient selection and treatment concepts. It summarizes available evidence from retro- and prospective studies evaluating SBRT alone, SBRT in combination with other treatments and SBRT compared to other local treatment approaches.

Key words: Hepatocellular carcinoma; Stereotactic body radiation therapy; Local-ablative treatment; Combination approaches; Mini-review

Core tip: Stereotactic body radiation therapy (SBRT) is an emerging treatment for hepatocellular carcinoma. It may be used as a sole treatment or in combination with other local therapies as well as a bridging strategy for patient awaiting transplants and results in excellent local control rates with low toxicity. This mini-review describes

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current concepts of SBRT and summarizes the available evidence evaluating SBRT alone, SBRT in combination with other treatments and SBRT compared to other local treatment approaches.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most common cancer worldwide and ranking as the third most common cause of cancer death[1]. Tumour resection or liver transplantation is the main curative treatment options. However, only a minority of patients are suitable candidates for surgical treatment due to major vascular involvement, large multifocal lesions or accompanying comorbidities such as poor liver function or associated problems[2]. In the past, inoperable cases have traditionally been regarded as incurable. Treatment paradigms have changed dramatically in favor of local treatments in the last decades though. Even in inoperable patients, there is now emerging evidence of survival benefit or potential cure in inoperable patients receiving local treatments[3,4]. In consequence, local therapies should be considered in patients not eligible for curative surgery, or as a part of a strategy to bridge patients awaiting liver transplantation according to common guidelines[5]. Local treatments are broadly classified into two categories: Arterially-directed and locally ablative therapies. Arterially directed therapies include transarterial chemoembolization (TACE), transarterial chemoembolisation with drug eluting beds (DEB-TACE), and selective internal radiotherapy (SIRT). Locally ablative techniques include radiofrequency ablation (RFA), percutaneous alcohol injection, microwave or (less invasive) Stereotactic body radiation therapy (SBRT). However, potential benefits of these treatments need to be weighed against the potential treatment-induced impairment of liver function or even liver failure especially in the presence of underlying liver disease as a primary cause of most primary hepatic malignancies[4]. All of these treatments also have limitations and appropriate patient selection is crucial to achieve positive outcomes: Patients with multiple comorbidities or inadequate liver function are usually poor candidates for surgical inverventions[4], patients with lesions directly adjacent to major vessels or bile ducts are not well suited for RFA[6], and patients with portal vein thrombosis rarely qualify for TACE or SIRT[7].

SBRT is an additional locally ablative treatment option for patients with HCC who are not eligible for resection or other local treatments. It can also be used to bridge waiting time in patients qualifying for transplantation or as part of multi-modality treatments with other liver- directed therapies[3]. In the absence of level I evidence, SBRT is not considered a standard in many guidelines, unfortunately. This mini-review describes current SBRT techniques and summarizes published evidence regarding efficacy and toxicity as a single treatment or in combination with other liver-directed therapies.

SBRT: INDICATIONS AND TECHNIQUES

SBRT is a highly conformal technique of external beam radiation therapy (EBRT) delivering high radiation doses in a small number of fractions[8]. Tumour control is achieved by high doses per fraction leading to high biological effectiveness and hence increased cell kill. Due to sharp dose gradients outside the target volume, dose to adjacent organs at risk is effectively limited maintaining adequate organ function. Stereotactic radiotherapy was initially developed for treatment of small cerebral lesions as stereotactic radiosurgery (SRS), the same principle was developed further in order to treat extracranial lesions (SBRT = stereotactic body radiation therapy). SRS and SBRT have now been widely accepted as standard of care for the treatment of limited brain or lung metastases as well as for early stage non-small-cell lung cancer. Clinical studies could show that SRS/SBRT and surgical approaches yield comparable results[8-10]. Meanwhile, SBRT is increasingly used for treatment of liver, lymph node or bony lesions[4,11,12].

In liver lesions, SBRT is usually indicated in patients with 1-3 lesions with a maximum diameter of 5-6 cm[13] who are not eligible for resection or other local therapies either as definitive or bridging therapy prior to transplantation[13]. Preservation of adequate liver function is mandatory, which is estimated individually based on total liver volume, lesion size and number, prior treatments and current liver function[4,13]. In general, patients with liver cirrhosis Child Pugh class A and early B are suitable candidates. In contrast to RFA/TACE treatment, patients with lesions located close to the liver surface, directly adjacent to large vessels, or portal vein thrombosis as well as patients presenting with extensive ascites are still candidates for SBRT. In contrast, However, patients with lesions directly adjacent to structures with low radiation tolerance like small bowel or stomach are less good candidates because dose reduction may be necessary[4,14,15].

Technically, SBRT is a form of precision external beam radiation therapy using minimal safety margins[8]. In consequence, accurate target delineation and treatment planning, precise patient positioning, careful image guidance and adequate motion management strategies are mandatory. Target delineation usually includes multi-modality imaging such as multi-phase contrast-enhanced computed tomography (CT) and magnetic resonance imaging preferably with liver-specific contrast-agents (see Figure 1). Patient positioning may include supportive vacuum pillows or other immobilization devices. Treatment planning is usually performed using multi-field or rotational techniques (see Figure 2). On-board imaging usually includes at least three-dimensional cone beam CT prior to each fraction. Unfortunately, HCCs are poorly visible in native CT scans and can therefore rarely be identified by linac-based imaging, hence perilesional placement of fiducials prior to treatment planning is commonly necessary[4,16,17]. Depending on respective SBRT strategy, 1-4 gold or platin markers are placed near the lesion under CT or ultrasound guidance. These markers can be easily identified with all common image-guidance procedures (especially cone beam-CT) and used for patient set-up as well as gating or tracking strategies[4]. Exceptions can be made if SBRT is applied shortly following TACE and there is still adequate contrast enhancement of lipiodol or if clips from prior surgical resections are present in direct proximity to the current lesions[3,4], (see Figure 2).

Apart from implantation of fiducial markers, SBRT represents a non-invasive treatment option. Motion mitigation may be managed by either internal target volume concepts (ITV) or gating/tracking strategies. In order to define the ITV, the lesion is delineated on different respiratory phases based on a contrast-enhanced four-dimensional CT. The ITV corresponds to the resulting enveloping volume, which includes each delineated lesion position during the respiratory cycle and can be treated without breathing control or gating. In patients with large respiratory excursions, abdominal compression devices may be used to reduce motion and therefore limit resulting absolute ITVs[18]. In gating strategies, lesion motion is either derived from continuous breathing detection by imaging or patient surface detection or continuously detected through electromagnetic transponders. Radiation is applied only during short phases of the breathing cycle when the specific lesion is within a specified position or corridor, tracking techniques model lesion motion with respect to the breathing cycle. Accuracy of the model is checked and corrected in real time feeding back to the treatment position. In consequence, the radiation beam moves with the target and according to the model utilizing the whole breathing cycle and thereby reducing overall treatment time as compared to gating strategies. Doses are typically prescribed to a lesion-surrounding isodose (*i.e.*, 65% or 80%), resulting in inhomogenous dose distributions. The lesion center therefore intentionally receives significantly higher doses while doses fall off quite sharply outside of the target volume. In consequence, doses and toxicities in adjacent normal tissue are reduced (see Figure 2). A variety of dose prescription and fractionation schedules have been employed. Currently most centers use 3-6 fractions of 8-20 Gy each, depending on localization, lesion size and liver function[4]. In order to preserve adequate liver function following SBRT, attention needs to be paid to specifying and sparing a threshold volume of uninvolved liver (usually 700 mL). In addition, excessive doses to luminal structures must be avoided by keeping a minimum distance (*i.e.*, 5mm) to the high-dose area within the lesion[4]. If adequately performed, acute side effects following SBRT are rare and generally mild. These include fatigue, transient elevation of liver enzymes or unspecific abdominal symptoms. Late toxicities may include radiation-induced liver disease resulting in impaired liver function, gastrointestinal side effects like ulceration or stenosis, biliary complications and rib fractures. However, high-grade toxicities were rare and usually lower than in comparable series using alternative locally-ablative techniques[19-21]. Close follow-up evaluations including repeated imaging (see Figure 3) are necessary in order to evaluate resultant toxicity and to detect early local or distant progression[3]. It is of note though that SBRT may induce several and characteristic types of tumor and surrounding tissue

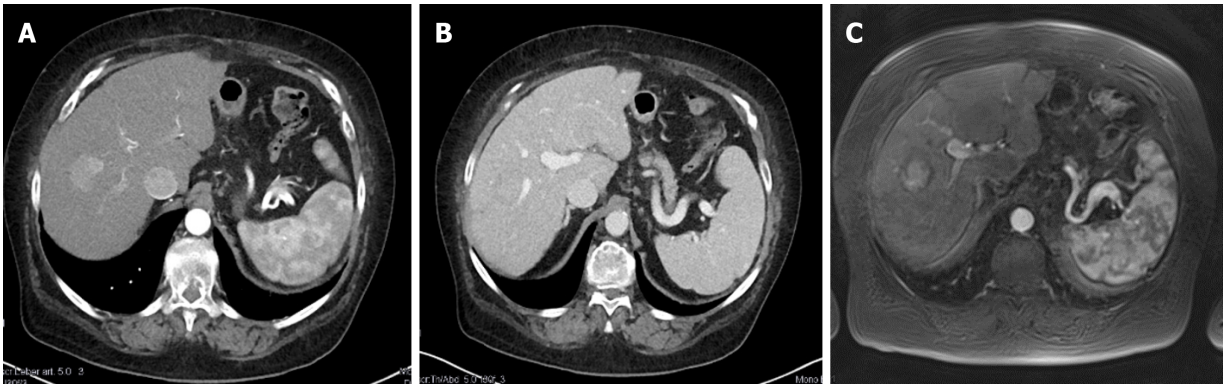

Figure 1 Hepatocellular carcinoma in segment VIII at diagnosis. A: Contrast-enhanced computed tomography (CT) arterial phase; B: Contrast-enhanced CT venous phase; C: Magnetic resonance imaging with liver-specific contrast agent.

alterations over time which should not be confused with progressive disease. For example, Herfarth *et al*[22] described three distinct types of focal reactions on multiphase contrast-enhanced CT following SBRT in their landmark paper. All of those are subject to substantial change over time and correlated to applied dose but have to be distinguished from disease recurrence. Lesions treated by SBRT may show signs of activity like hypervascularisation, wash-out or absence of regression in size up to 12 mo after treatment without residual viable tumor as reported by Mendiratta-Lala *et al*[23]. Tétreau *et al*[24] compared different criteria for response evaluation and found that RECIST (Response evaluation criteria in solid tumors) criteria were unsuitable for response assessment and were outperformed by EASL (European Association of Study of the liver) criteria at each point of time during available follow-up. Therefore, response assessment including decision-making for salvage treatments following SBRT should preferably be made by a multidisciplinary panel including experienced radiation oncologists.

SBRT: CLINICAL EVIDENCE

In recent years an increasing number studies have been published, including mainly small retrospective cohorts but also larger series and well-designed phase II trials, see Table 1. Comparison of published data is hampered by varying and inhomogenous inclusion criteria across these studies. In addition, most series include large numbers of patients/lesions receiving SBRT because they were not eligible for other local treatments options (anymore) and/or have been treated with other techniques multiple times before. In consequence, most SBRT series represent a negative pre-selection of patients ex ante as compared to series reporting on other local treatments mainly as the primary treatment option. Nevertheless, SBRT resulted in very encouraging local control (1-year LC 65%-100%) and overall survival rates (1-year OS 32%-94%) with low toxicity[14,25-39]. In addition to dose and fractionation[28,30,34], local control appears to be determined by lesion size[28,34] and number of lesions[1], while overall survival is strongly associated with general condition and liver function prior to treatment. Several groups have consistently shown clear survival benefits after SBRT in Child-Pugh class A (CP-A) patients when compared to CP-B patients[14,27,34,39]. CP-B patients further suffered from significantly increased toxicity despite receiving lower SBRT doses and less aggressive fractionation schemes[14,27], thus possible benefits and risks of SBRT have to be considered carefully when selecting those patients for treatment.

Direct comparisons of SBRT with other local treatment options are limited and analyses most commonly retrospective (see Table 2). Su *et al*[40] compared SBRT with surgery in a propensity score matched cohort. Only patients with adequate liver function (CP-A), relatively small lesions (median 3.3 cm) treated in primary situation were included in the analysis. Despite mature follow-up of these cohorts, the authors could not detect significant differences between these treatment modalities with regard to either local control or overall survival. However, they described significant differences in accompanying toxicity profiles. While surgically treated patients showed less nausea, SBRT patients suffered less often from bleeding and pain. Wahl *et al*[19] performed a retrospective comparison of SBRT and RFA in a series of 224 patients. Except for a distinctly higher rate of prior treatments in the SBRT group, both arms seemed comparable with respect to major prognostic factors. Again, no

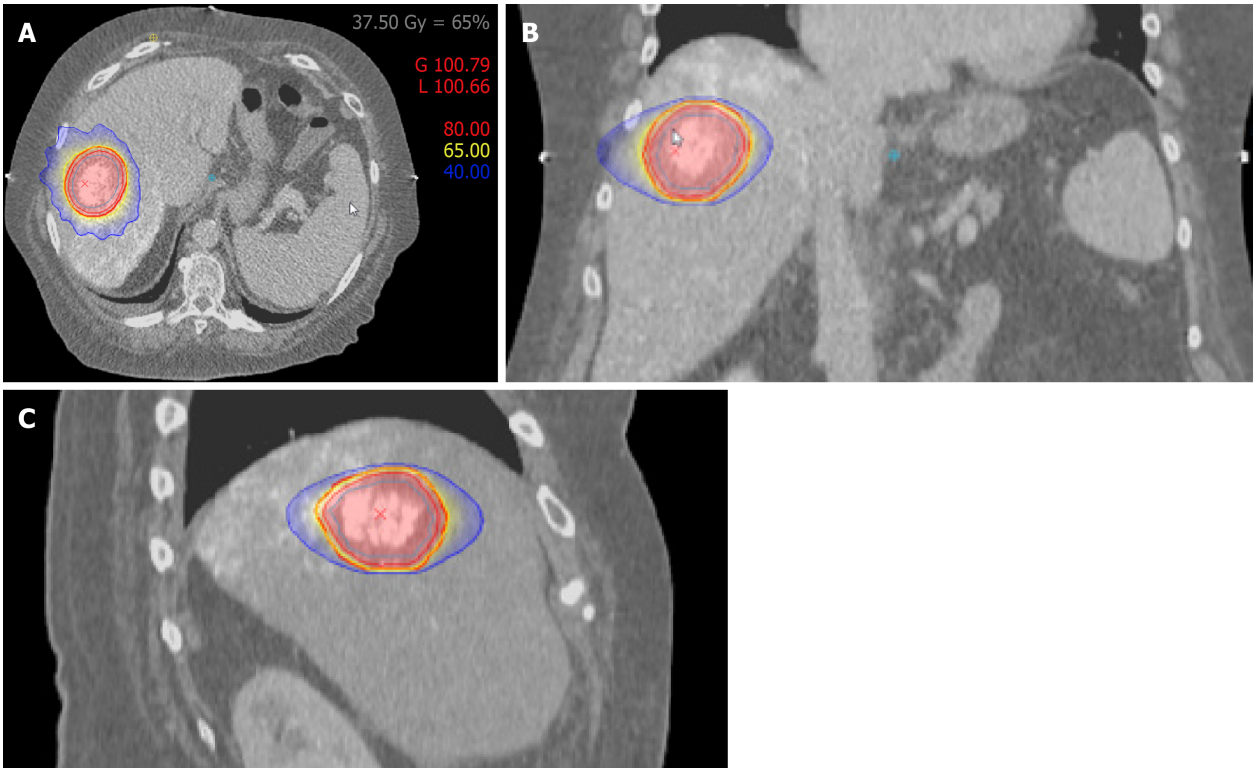

Figure 2 Treatment plan (prescription dose 3 × 12.5 Gy to 65% surrounding isodose). A: Isodose plan in axial view; B: Frontal view; C: Sagittal view, broad red line: Planning target volume (PTV), yellow line: PTV-surrounding 65% isodose = 37.5 Gy, light blue line: Internal target volume (ITV), narrow red line: ITV-surrounding 80% isodose = 46.2 Gy, dark blue line: 40% isodose = 23.1 Gy.

significant difference in local control and overall survival was found between the cohorts. While both treatment were similarly efficient in lesions < 2 cm, the analysis showed significantly improved local control in patients treated with SBRT for larger lesions[19]. Sapir *et al*[20] compared SBRT with TACE in a retrospective series including 209 patients. Both groups were comparable with respect to their baseline characteristics with two exceptions: patients in the SBRT group were more heavily pre-treated, while mean lesion diameter was higher in the TACE group. Keeping those limitations in mind, SBRT resulted in significantly increased local control (1-year LC 97% *vs* 47%) and favourable toxicity profile although this benefit did not translate into a clear survival benefit (1-year OS 75% *vs* 74%)[20].

In summary, SBRT seems to result at least in similar local control and overall survival rates as compared to other local treatments while showing mainly favorable toxicity profiles based on currently available albeit limited evidence. Therefore, SBRT may represent a reasonable alternative to other local treatments and should be considered as potential treatment modality in multidisciplinary evaluations of suitable patients.

SBRT COMBINED WITH OTHER TREATMENTS

RFA/TACE

Combination of SBRT with other local therapies for treatment of either the same or different lesions may result in synergistic effects[3]. In case of multifocal disease with several lesions of various sites and size, some lesions may be easily addressed by RFA while others (*i.e.*, due to close proximity to major vessels) may profit from SBRT. When combining different approaches, invasive procedures should be scheduled first, as fiducials (which are often necessary for SBRT) can be implanted in the same session without risks of an additional intervention.

Combining TACE with SBRT in the treatment of the same lesion may offer several advantages (see Figure 1-3). Prior TACE may result in tumour response and hence smaller SBRT volume leading to potentially improved toxicity[4]. Chemotherapy as a component of TACE may act as a radiosensitizer also enhancing the radiation effect of SBRT[4], although this might be counterbalanced by tumor hypoxia induced by embolization. Lipiodol deposits placed during embolization can also serve as

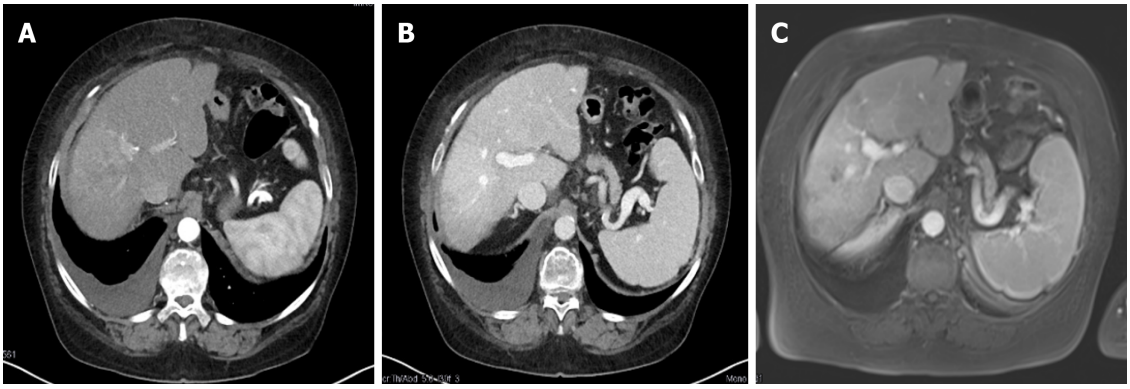

Figure 3 Complete response 9 months after transarterial chemoembolization and stereotactic body radiation therapy. A: Contrast-enhanced computed tomography (CT) arterial phase; B: Contrast-enhanced CT venous phase; C: Magnetic resonance imaging with liver-specific contrast agent.

landmarks for image guidance in SBRT, which may potentially render fiducial placement unnecessary[3,4]. Indeed, small retrospective series have shown significant improvements regarding treatment response, local control, progression-free survival, and even overall survival by the addition of SBRT to TACE compared to TACE alone at least if lesion size exceeded 3 cm[41,42]. Kang *et al*[28] reported a prospective phase II trial using SBRT following TACE. Fifty patients with lesion size < 10 cm and CP-A or early CP-B cirrhosis were enrolled. Patients received SBRT in 3 fractions with 14-20 Gy per fraction. The group reported very encouraging 2-year local and overall survival rates of 95% and 69%. Toxicities of grade III or higher were observed in only 10% despite comparatively high doses. In summary, combination of TACE and SBRT seems to be a very promising approach, which is currently evaluated in several prospective trials.

Sorafenib

Although preclinical data suggested radiation-sensitizing effects of sorafenib[43], combination of Sorafenib with SBRT does not appear advisable. Prospective clinical trials reported discouraging toxicities: Brade *et al*[44] conducted a phase I trial investigating SBRT with concurrent Sorafenib in CP-A patients unsuitable for standard local therapies. Nine out of 16 patients showed grade 3+ toxicity including 2 deaths. While 15 of 16 patients completed SBRT as planned, adherence to Sorafenib treatment was poor: Only 3 out of 16 patients completed treatment for the first 12 wk without modifications. The authors concluded that concurrent use of SBRT and Sorafenib should not be recommended. Based on the preclinical data they advocated in favor of evaluating a sequential approach, which is currently under investigation within a randomized trial (RTOG 112).

SBRT AS BRIDGING TO TRANSPLANT

Many patients who were initially eligible for liver transplantation unfortunately drop off waiting lists due to tumour progression. As a result, increasing attention is paid to bridging approaches to reduce this number. Based on limited evidence from retrospective analyses, SBRT seems to be a reasonable option. For example, Katz *et al*[45] reported 18 patients treated with SBRT as bridging approach. All patients received 50 Gy in 10 fractions. 6 patients were delisted due to various reasons while the remaining 12 finally received major surgery or transplant after a median of 6.3 months. No grade 3+ toxicities were reported. Pathologic complete response rate in explanted organs following SBRT was 20%. Local control until transplantation was achieved in all patients. With a median follow-up of 20 mo, all patients are disease-free and alive. O´Connor *et al*[46] similarly described a series of 11 patients with median lesion size of 3.4 cm who received SBRT with 33-54 Gy in 3 fractions as bridging. Patients underwent liver transplantation after a median interval of 113 d. Again no patient experienced grade 3+ toxicity. Pathologic complete response was found in 27% and 5-year DFS and OS after transplantation were 100%. Interestingly, patients receiving 54 Gy in 3 fractions showed a distinctly higher pathologic complete response rate of 60%. Mohamed *et al*[21] evaluated various bridging strategies including RFA, TACE, SBRT and SIRT. They found high pathologic complete response rates for all bridging treatments but noticed favorable toxicity profiles for SBRT and SIRT (no grade 3+ toxicity). Finally, Murray *et al*[4] noted in a recent review

Table 1 Prospective trials and large (> 100 patients) retrospective series evaluating stereotactic body radiation therapy in hepatocellular carcinoma

Author	Yr	Type	*n*	Size	VI	PVT	mf	PT	CP class	f/u	Dose	1y-LC	1y-OS
Méndez Romero *et al*[25]	2006	phase I/II	8 (11)	3.5 (0.5-7) cm	38%	25%	25%	NR	A: 63%, B: 25%, UK: 12%	13	25-37.5/3-5Fx	75%	75%
Tse *et al*[26]	2008	phase I	31 (NB)	173 (9-1913) mL	52%	NR	NR	61%	A: 100%	18[1]	24-54/6Fx	65%[1]	48%
Cárdenes *et al*[27]	2010	phase I	17 (25)	34 (8-95) mL	NR	18%	30%	24%	A: 35%, B: 65%	24	36-48/3-5Fx	100%	75%
Kang *et al*[28]	2012	phase II	47 (56)	15 (2-214) mL	NR	29%	17%	100%[2]	A: 87%, B: 13%	17	42-60/3Fx	95%[4]	69%[4]
Price *et al*[29]	2012	phase I/II	26 (29)	NR (21-253) mL	NR	12%	12%	27%	A: 54%, B: 46%	13	36-48/3-5Fx	96%	77%
Huang *et al*[30]	2012	phase II	36 (NB)	4.8 (1.1-12.3) cm	NR	NR	NR	NR	A: 78%, B: 19%, C: 3%	14	25-48/4-5Fx	88%	64%[4]
Bujold *et al*[31]	2013	phase I/II	102 (NB)	117 (1-1913) mL	55%	NR	61%	52%	A: 100%	31	24-54/6Fx	87%	55%
Culleton *et al*[32]	2014	phase II	29 (NB)	9 (4-27) cm	NR	76%	NR	14%	B: 97%, C: 3%	NR	21-49/5-15Fx	NR	32%
Sanuki *et al*[33]	2014	retro	185 (185)	8 (1.6-65) mL	NR	NR	0%	68%[2]	A: 85%, B: 15%	23	35-40/5Fx	99%	95%
Lasley *et al*[14]	2015	phase I/II	59 (65)	34 (2-107) mL	NR	NR	NR	NR	A: 64%, B: 36%	33/46[3]	36-48/3-5Fx	NR	91%/82%[3]
Scorsetti *et al*[34]	2015	phase II	43 (63)	5 (1-13) cm	NR	20%	43%	65%	A: 53%, B: 47%	8	36-75/3-6Fx	86%	78%
Su *et al*[35]	2016	retro	132 (175)	3 (1.1-5) cm	NR	NR	28%	30%	A: 86%, B: 14%	21	42-46/3-Fx	91%	94%
Takeda *et al*[36]	2016	phase II	90 (90)	NR (1-4) cm	NR	NR	0%	64%	A: 91%, B: 9%	42	35-40/5Fx	96%[5]	67%[5]
Moon *et al*[37]	2018	phase II	11 (NB)	23 (3-145) mL[1]	NR	NR	13%[1]	48%[1]	NR	13[1]	27.5-45/3-5Fx	82%	36%
Nabavizadeh *et al*[38]	2018	retro	146 (146)	NR	NR	10%	0%	92%	A: 46%,B: 41%,C: 13%	23	50/5Fx[6]	97%	NR
Jeong *et al*[39]	2018	retro	119 (139)	1.7 (NR) cm	0%	0%	NR	97%	A: 91%, B: 9%	26	30-60/3Fx	99%	99%

[1]All patients (including different histologies);
[2]TACE 1-2 mo prior to SBRT;
[3]Reported separately for CP-A and CP-B patients;
[4]2-year rate;
[5]3-year rate;
[6]Patients with poor liver function were treated with hypofractionated radiation therapy (45 Gy in 18 fractions).
n: Number of patients (lesions); cm: Cm diameter; mL: Milliliter volume; VI: Vascular invasion; PVT: Portal vein thrombosis; mf: Multifokal; PT: Prior treatment; CP: Child-Pugh; f/u: Median follow-up in months; dose: Total dose in Gy; Fx: Number of fractions; 1y-LC: 1-year local control rate; 1y-OS: 1-year overall survival rate; retro: Retrospective; UK: Unknown; NR: Not reported.

that 63%-100% of patients treated with SBRT as bridging proceeded to transplantation with explants showing pathologic complete and partial responses in 14%-27% and 23%-64% of lesions.

In summary, SBRT seems to be another suitable option to bridge patients scheduled for liver transplant, which shows similar response rates but very modest toxicity profiles as compared to other local treatment options and should be considered in the multidisciplinary evaluation.

FUTURE DIRECTIONS

Future developments regarding SBRT mainly focus on MRI-based treatment planning followed by real-time MRI-guided radiation therapy. The implementation of daily image guidance and replanning using MR-linac technology with enhanced soft-tissue information may not only result in increased set-up accuracy. It may however, allow omission of fiducial placement prior to SBRT, thus rendering SBRT a completely non-invasive treatment option. Furthermore, particle therapy (protons or heavy ions) seems to be a promising option due to higher biological effectiveness (heavy ions) and dosimetric advantages. However, the main benefit of protons (the lack of exit dose) may be offset in liver tumors by several factors: Meticulous motion mitigation techniques are crucial in order to minimize range uncertainties caused by moving air-soft-tissue or air-bony interface. Air-filled cavities in adjacent luminal organs present

Table 2 Studies comparing stereotactic body radiation therapy to other local treatments

Author	Yr	Type	Treat.	*n*	Size	mf	PT	CP class	f/u	Dose	1y-LC	1y-OS	tox.	Comment
Su *et al*[40]	2017	pm	SBRT	33 (45)	3.3 (NR) cm	36%	0%	A: 100%	42	42-48/3Fx	84%[1]	100%	nausea[4]	LC/OS NS
			OP	33 (45)	3.3 (NR) cm	30%	0%	A: 100%	44		72%[1]	97%	bleed./pain[5]	
Wahl *et al*[19]	2016	retro	SBRT	63 (83)	2.2 (0.1-10) cm	29%	2 (0-7)[2]	A: 69%, B: 29%, C: 2%	13	30-50/3-5Fx	97%	74%	grade3+: 3%	LC/OS NS
			RFA	161 (249)	1.8 (0.6-7) cm	32%	0 (0-7)[2]	A: 50%, B: 42%, C: 8%	20		84%	70%	grade3+: 11%	> 2 cm LC sig↑ with SBRT
Sapir *et al*[20]	2018	retro	SBRT	125 (173)	2.3 (0.1-20.8) cm	NR	2 (NR)[2]	6 (5-9)[3]	12	30-50/3-5Fx	97%	75%	grade3+: 8%	LC sig↑ with SBRT
			TACE	84 (84)	2.9 (0.7-15) cm	NR	0 (NR)[2]	6 (5-9)[3]	23		47%	74%	grade3+: 13%	Tox sig↑ with TACE

[1]Intrahepatic recurrence free survival;
[2]Number of prior treatments median (range);
[3]CP score median (range);
[4]All grades, significantly increased with SBRT;
[5]All grades, significantly increaesd with surgery.
treat.: Treatment; *n*: Number of patients (lesions); size: Lesion size median(range); cm: Centimeter diameter; mf: Multifokal; PT: Prior treatment; CP: Child-Pugh; f/u: Median follow-up in months; dose: Total dose in Gy; Fx: Number of fractions; 1y-LC: 1-year local control rate; 1y-OS: 1-year overall survival rate; tox: Toxicity, NS: Not significant; pm: Propensity score matched pair analysis; retro: Retrospective; bleed.: bleeding; sig: Significant; OP: Surgery; RFA: Radiofrequency ablation; TACE: Transarterial chemoablation; NR: Not reported.

further challenges[47]. Nevertheless, several reports describing early experiences with protons have shown high local control rates and low toxicities[48,49]. The potential benefit is currently evaluated in a phase III trial (NRG-GI003) comparing photon and proton SBRT in unresectable HCC[47].

CONCLUSIONS

Evidence comparing various strategies for the treatment of HCC is limited. Based on available data, SBRT is an effective treatment option for HCC accompanied by low rates of toxicity. Outcomes seem at least comparable to other local treatment options or limited (non-transplantation) surgical approaches. Combination with other local therapies especially TACE appears to be feasible and seems to result in synergistic effects. SBRT may also be reasonably used as a bridging option in patients awaiting liver transplantation. Dose and fractionation should be prescribed individually based on liver volume, lesion size and number, prior treatments, current liver function and adjacent organs at risk and adequate patient selection is crucial.

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ORIGINAL ARTICLE

Basic Study

Qingjie Fuzheng granules inhibit colorectal cancer cell growth by the PI3K/AKT and ERK pathways

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Author contributions: Lin JM and Yang H conceived and designed the experiments; Yang H, Liu JX and Shang HX conducted MTT, LDH, Hoechst 33258 and western blot assays and analysis; Lin S and Zhao JY conducted fluorescence-activated cell sorting and analysis; Yang H and Lin JM wrote the manuscript. All authors are responsible for the obtainment of written permission to use any copyrighted text and/or illustrations.

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Abstract

BACKGROUND

Qingjie Fuzheng granules (QFGs) are part of a traditional Chinese medicine formula, which has been widely used and found to be clinically effective with few side effects in various cancer treatments, including colorectal cancer (CRC). However, the precise mechanisms and molecular signaling pathways involved in the activity of QFGs' anticancer effect have not been reported in the literature. In this study, we hypothesized that QFGs can inhibit the growth of colorectal cancer cells, and that its mechanism is closely related to one or more intracellular signal transduction pathways.

AIM

To better evaluate the mechanism underlying the anti-cancer effect of QFGs on the CRC cell lines HCT-116 and HCT-8.

METHOD

First, we measured cell viability and cytotoxicity by performing MTT and lactate dehydrogenase (LDH) assays. We evaluated the role of QFGs in cell proliferation and apoptosis by assessing colony formation and analyzing Hoechst 33258 staining. Second, cell cycle and apoptosis rates were measured by fluorescence activated cell sorting, and the expression levels of survivin, cyclin D1, CDK4, p21, Bax, Bcl-2, Fas, FasL, and cleaved-caspase-3/-8/-9 were measured by performing western blots and caspase activity assays. Furthermore, inhibitors of caspase-3/-8/-9 were used to elucidate the specific apoptosis pathway induced by QFGs in cancer cells. Finally, activation of the PI3K/AKT and ERK signaling pathways

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was examined using the western blot assay to investigate the possible mechanism.

RESULTS
MTT and LDH assays revealed that after 0.5-2.0 mg/mL of QFGs treatment, cell viability was reduced by (6.90% ± 1.03%)–(59.70% ± 1.51%) (HCT-116; $P < 0.05$) and (5.56% ± 4.52%)–(49.44% ± 2.47%) (HCT-8; $P < 0.05$), and cytotoxicity was increased from 0.52 ± 0.023 to 0.77 ± 0.002 (HCT-116; $P < 0.01$) and from 0.56 ± 0.054 to 0.81 ± 0.044 (HCT-8; $P < 0.01$) compared with the non-QFGs treatment groups. Additionally, colony formation and Hoechst 33258 staining assays showed that QFGs inhibited proliferation and induced apoptosis in CRC cells. QFGs also increased the expression levels of Bax, Fas and FasL, decreased the level of Bcl-2, and stimulated the activation of caspase-3/-8/-9, which were revealed by western blot and caspase activity assays. In contrast, when adding the three caspase inhibitors, the suppression effect of QFGs on cell viability and apoptosis were markedly inhibited. Moreover, QFGs suppressed the phosphorylation levels of PI3K, AKT and ERK.

CONCLUSION
These results demonstrated that QFGs can inhibit CRC cell proliferation and induce apoptosis by suppressing the PI3K/AKT and ERK signaling pathways.

Key words: Qingjie Fuzheng granules; Colorectal cancer; Proliferation; Apoptosis; PI3K/AKT; ERK

Core tip: To study the effect and mechanism of Qingjie Fuzheng granules (QFGs) on colorectal cancer (CRC) cells, we measured cell viability and cytotoxicity by performing MTT and LDH assays. We also evaluated the role of QFGs in cell proliferation by conducting colony formation, cell cycle and western blot assays. We evaluated the role of QFGs in cell apoptosis by assessing both Hoechst 33258 and Annexin-V/PI staining and performing western blot assays. Furthermore, the activation of PI3K/AKT and ERK signaling pathways was examined using the western blot assay to investigate the possible mechanism. All results demonstrated that QFGs inhibited CRC cell growth by suppressing the PI3K/AKT and ERK signaling pathways.

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INTRODUCTION

As a result of changing lifestyles and aging populations, the prevalence of colorectal cancer (CRC) remains high and accounts for approximately 25% of the world's cancer-related deaths[1-3]. Although surgical resection and chemoradiotherapy are the most commonly used clinical options, surgery is not an option for all patients, and long-term chemoradiotherapy can cause adverse side effects, such as drug resistance, recurrence and metastasis[4-7]. Therefore, the search for novel therapies has attracted worldwide attention.

Natural products, which often have fewer side effects than synthetic drugs, are important in the treatment of many diseases and have a long history of use in China. Therefore, natural products have been studied by many researchers to find better antitumor drugs[8-11]. Qingjie Fuzheng granules (QFGs) is a traditional Chinese medicine (TCM) formula (Table 1) that consists of a mixture of four herbs (*Scutellaria barbata* D. Don, malt, *Hedyotis diffusa* Willd, and Astragalus) that together confer properties of anti-inflammation, antioxidative, antibacterial, immunity enhancement and digestion promotion. QFGs have been widely used and found to be clinically effective in various cancer treatments, including CRC, and have few side effects.

However, the precise mechanisms and molecular signaling pathways involved in the activity of QFGs' anticancer effect have not been reported in the literature.

CRC develops because of a cell growth imbalance caused by excessive proliferation or lack of apoptosis. Eukaryotic cell proliferation is controlled by the cell cycle, which consists of the G0, G1, S, G2 and M phases. In the detection of cell cycle progression, the G1/S transition is one of the main checkpoints[12]. The main regulatory factors in G1/S progression are cyclin D1 and cyclin-dependent kinase 4 (CDK4), which can form complexes to regulate this progress[13-15]. A CDK inhibitor, p21, can change the function of CDK–cyclin complexes by binding to them and then suppressing cell proliferation[16]. Normal cell apoptosis can eliminate surplus, redundant, and aberrant cells in animals, so it is essential for normal tissue maintenance. Disorders in this process trigger many diseases, including CRC[17-19]. The pathways involved in the apoptotic process are the mitochondria-dependent pathway, also called the intrinsic apoptosis pathway, and the death receptor-mediated apoptosis pathway[20]. The former is modulated by the Bax (proapoptotic) and Bcl-2 (anti-apoptotic) family proteins[21], which control the release of apoptotic correlation factors, such as cytochrome C (Cyt C)[22]. When intracellular damage occurs, mitochondria-dependent apoptosis is triggered. Then, Cyt C, together with Apaf-1 and caspase-9, cleaves caspase-3[23]. Receptor-mediated apoptosis originates from outside the cell, with the binding of the Fas ligand (termed FasL or CD95L) to the Fas receptor (termed CD95). Once the death receptor pathway is successfully activated, the Fas-associated death domain and caspase-8 will accumulate, and caspase-8 will be cleaved. Then, caspase-8 cleaves caspase-3, which generates the activated form of caspase-3 that serves as the ultimate activator of apoptosis[24]. Therefore, one of the key approaches in the development of antitumor drugs is to promote apoptosis and inhibit tumor cell proliferation, two processes that typically promote cancer growth. There are multiple signaling pathways that regulate cancer growth, including the PI3K/AKT and ERK signaling pathways, and abnormal activation of these signaling pathways can lead to irregular expression of these factors.

The aim of this study is to better understand the mechanism underlying the potential anticancer effect of QFGs by investigating their biological function using the human CRC cell variants HCT-116 and HCT-8. Our results showed that QFGs inhibit proliferation and increase apoptosis in HCT-116 and HCT-8 cells by inactivating the PI3K/AKT and ERK pathways.

MATERIALS AND METHODS

Cell culture

The human colon carcinoma HCT-8 and HCT-116 cell lines were purchased from the American Type Culture Collection. The two cell lines were cultured in Roswell Park Memorial Institute-1640 medium (C11875500BT; Life Technologies Corp. Grand Island, United States) containing 10% fetal bovine serum, 1% penicillin, and 1% streptomycin, and were grown at 37 °C in 5% CO_2.

Preparation of QFGs and caspase inhibitors

QFGs were obtained and prepared as previously described[11]. Briefly, QFGs powder was dissolved in 1× PBS (store concentration of QFGs is 200 mg/mL) and stored at 20 °C. Inhibitors of caspase-3/-8/-9 (Z-DEVE-FMK, ab120488; Z-IETD-FMK, ab141382; Z-LEHD-FMK, ab142026, Abcam, CA, United States) were dissolved in DMSO to a concentration of 10 mM and stored at -20 °C.

MTT assays

HCT-8 and HCT-116 cells were placed into 96-well plates (1×10^5 cell/well). After 12 h, the cells were treated with different doses of QFGs (0.5, 1 and 2 mg/mL) and grown for 24 h, or the cells were treated with a designated dose of QFGs (2 mg/mL) in combination with inhibitors of caspase-3 (Z-DEVE-FMK), caspase-8 (Z-IETD-FMK), and caspase-9 (Z-LEHD-FMK) at a concentration of 10 μmol/L each and then incubated for 24 h. Then, MTT (0.5 mg/mL) was added to each well (100 μL/well) and incubated for 4 h. Subsequently, all wells were treated with DMSO (100 μL/well). Absorbance at 570 nm in each well was measured by using an ELISA reader (Infinite M200 PRO; Tecan Austria GmbH, Austria).

Lactate dehydrogenase assays

Cells were seeded into 12-well plates (1×10^5 cell/well), treated with different dose of QFGs (0.5, 1 and 2 mg/mL) and grown for 24 h. Then, a lactate dehydrogenase (LDH) release assay kit (Beyotime, Shanghai, China) was used to determine the LDH activity according to the kit's manual.

Table 1 Composition of Qingjie Fuzheng granules

Common name	Latin name	Part used	Daily adult dose, g
Spreading Hedyotis herb	*Hedyotis diffusa* Willd	Dried root	15
Malt	*Hordeum vulgare* L.	Dried seed	15
Astragalus	*Radix astragali*	Dried root	15
Scutellaria barbata	*Scutellaria barbata* D. Don	Dried body	15

Colony formation assays
After treatment with different concentrations of QFGs (0.5, 1, and 2 mg/mL) for 24 h, a colony formation assay was performed as described previously[11].

Hoechst 33258 staining
QFGs and 10 μmol/L caspase inhibitors (Z-DEVE-FMK, Z-IETD-FMK, Z-LEHD-FMK) were added to the cells and grown for 24 h. Subsequently, 4% paraformaldehyde was used to fix the cells for 15 min. Then, 4% paraformaldehyde was removed and 1× PBS was used to rinse the cells three times. Then, Hoechst 33258 (c0003; Beyotime, Shanghai, China) (100 μL/well) was added to all wells in the dark for 15 min. The Hoechst 33258 solution was then removed, and 1× PBS was used to rinse the stained cells three times, followed by 1× addition of fresh PBS. An inverted fluorescence microscope (Leica DMI4000B; Leica Camera AG, Solms, Germany) was used to observe and photograph the cells.

Cell cycle assays
Cell cycle was measured in the HCT-8 and HCT-116 cells after treatment with the indicated concentrations of QFGs (0.5, 1 and 2 mg/mL). Cell cycle progression was estimated by using a propidium iodide (PI) kit (KGA512; KeyGen Biotech, Nanjing, China) and fluorescence activated cell sorting according to the manufacturer's instructions.

Annexin V-FITC/PI staining flow cytometry assays
Cells were seeded into 6-well plates (1.5×10^5 cell/well), treated with different doses of QFGs (05, 1 and 2 mg/mL) and grown for 24 h. Then, cells were stained by using an Annexin V/PI kit (KGA108; KeyGen BioTech, Nanjing, China) according to the kit's manual. Annexin V-positivity and PI-negativity (lower-right quadrant) represented early apoptotic cells, whereas Annexin V-positivity and PI-positivity (upper-right quadrant) represented late apoptotic cells.

Caspase activity assays
A caspase activity assay kit (caspase-3, KGA204; caspase-8, KGA304; caspase-9, KGA404; KeyGen BioTech, Nanjing, China) was used to detect the activity of caspases according to the manufacturer's instructions. In brief, cell lysates were prepared after the addition of the indicated reaction buffer (provided in the kit) at 37 °C for 4 h in the dark. Absorbance at 405 nm was measured by using an ELISA reader.

Western blot analysis
When CRC cells were treated with different doses of QFGs (05, 1 and 2 mg/mL) for 24 h, a cell lysis buffer containing a cocktail to lyse the cells was added. The bicinchoninic acid assay was used to detect the total protein concentrations, and 50 μg of total protein was used for electroblotting. Five percent skim milk was used to block the NC membranes, and then primary antibodies against Fas, FasL, p-PI3K and p-AKT (ab-110021, ab-15285, ab182651, ab38449; 1:1000, Abcam, CA, United States), p-ERK (sc-16982; 1:1000, Santa Cruz Biotechnology, CA, United States), cleaved-caspase-3, cleaved-caspase-8, cleaved-caspase-9, β-actin, Bcl-2 and Bax (#9662, #4790, #9508, #4967, #4223, #5023; 1:1000, Cell Signaling, Beverly, MA, United States), PI3K, AKT and ERK (13329-1-AP, 10176-2-AP, 16443-1-AP; 1:2000, Proteintech, United States) were added at 4 °C overnight. On the second day, the appropriate HRP-conjugated secondary antibodies (goat anti-mouse IgG secondary antibody, #L3032; goat anti-rabbit IgG secondary, #L3012; 1:5000, Signalway Antibody, PA, United States) were added and the SuperSignal West Pico Chemiluminescent Substrate was used to detect the signal.

Statistical analysis
One-way ANOVA and SPSS software (version 18.0) were used to analyze all of the data in this study. The data are expressed as the mean ± standard deviation. $P < 0.05$

indicated statistical significance.

RESULTS

QFGs decreased cell viability and increased cytotoxicity in HCT-116 and HCT-8 cells

The MTT assays and LDH activity assays were used to evaluate the effect of QFGs on the growth of the two cell types. QFGs inhibited cell viability in a dose-dependent manner (Figure 1A, B), showing cytotoxicity at 0.5-2.0 mg/mL (Figure 1C, D). In Figure 1A and B, cell viability after treatment with QFGs (0.5–2.0 mg/mL, 24 h) decreased by (6.90% ± 1.03%)-(59.70% ± 1.51%) (HCT-116) and (5.56% ± 4.52%)-(49.44% ± 2.47%) (HCT-8) relative to the viability in control cells ($P < 0.05$). In Figure 1C and D, treatment with QFGs (0.5–2.0 mg/mL, 24 h) increased the LDH activity rate of the cells from 0.52 ± 0.023 to 0.77 ± 0.002 (HCT-116) and from 0.56 ± 0.054 to 0.81 ± 0.044 (HCT-8) relative to that in control cells ($P < 0.01$). These results proved that QFGs treatment reduced cell viability and increased cytotoxicity in both cell types.

QFGs inhibited the proliferation of HCT-116 and HCT-8 cells by arresting the cell cycle

Cell colony formation assays were used to evaluate the changes in cell growth after treatment with QFGs. As shown in Figure 2A, QFGs dose-dependency inhibited colony formation in HCT-116 and HCT-8 cells. Subsequently, cell cycle assays were used to verify the proliferation-inhibiting effects of QFGs. As shown in Figure 2B-E, the percentages of S phase in HCT-116 cells after treatment with 0, 0.5, 1, and 2 mg/mL QFGs were 44.7% ± 2.77%, 33.45% ± 3.30%, 16.50% ± 2.12%, and 12.86% ± 2.51%, respectively ($P < 0.01$), and the percentages of S phase in HCT-8 cells after treatment with 0, 0.5, 1, and 2 mg/mL QFGs were 44.55% ± 3.32%, 26.71% ± 2.17%, 25.60% ± 2.19%, and 21.99% ± 3.30%, respectively ($P < 0.01$). These results suggested that QFGs can inhibit proliferation of both cell types by arresting the cell cycle.

QFGs induced apoptosis of HCT-116 and HCT-8 cells via the mitochondria-dependent and death receptor pathways

Hoechst 33258 staining assays were used to evaluate the changes in cell nuclear morphology after treatment with QFGs (Figure 3A). The degree of staining was low in untreated cells, but it gradually increased in the other three groups, which indicated a gradual increase in apoptosis. Subsequently, Annexin V-FITC/PI assays were used to verify the apoptosis-inducing effect of QFGs. As shown in Figure 3B, apoptosis percentages in HCT-116 cells after treatment with 0, 0.5, 1, and 2 mg/mL QFGs were 4.07% ± 0.48%, 11.87% ± 0.5%, 12.77% ± 0.67%, and 31.13% ± 0.73%, respectively ($P < 0.01$), and apoptosis percentages in HCT-8 cells after treatment with 0, 0.5, 1, and 2 mg/mL QFGs were 2.23% ± 0.50%, 9.34% ± 0.69%, 17.19% ± 0.55%, and 33.93% ± 0.93%, respectively ($P < 0.01$). We also found that QFGs upregulated the expression of cleaved-caspase-3/-8/-9 in both cell types (Figure 3C, D; $P < 0.01$). At the same time, the caspase activity was measured using a commercial caspase activity assay kit. Identical to the western blot results, the activities of caspase-3/-8/-9 were significantly enhanced by QFGs treatment in both cell types (Figure 3E, F; $P < 0.01$). These results indicated that QFGs induced apoptosis in the two cell types, and suggested that apoptosis occurred *via* both the mitochondria-dependent and death receptor-mediated pathways.

To further confirm these findings, various specific caspase inhibitors were used. As shown in Figure 4A, Z-DEVD-FMK, Z-IETD-FMK, and Z-LEHD-FMK markedly inhibited the inhibitory effect of QFGs on cell viability in both cell types ($P < 0.05$ and 0.01, respectively). In addition, we used the Hoechst 33258 staining assay to detect nuclear morphological changes, and all three caspase inhibitors clearly inhibited the apoptosis-induced effect of QFGs (2 mg/mL) in both cell types (Figure 4B). These findings proved that QFGs induced apoptosis *via* the mitochondria-dependent and death receptor-mediated pathways in both HCT-116 and HCT-8 cells.

QFGs regulated the expressions of survivin, cyclin D1, CDK4, p21, Bax, Bcl-2, Fas, and FasL in HCT-116 and HCT-8 cells

During cell cycle regulation, survivin is a key protein that indicates cell cycle progression, and the complex of cyclin D1 and CDK4 directly regulates cell cycle progression[13]. In a previous study, overexpression of this complex has been found to induce cell proliferation, whereas p21 inhibited the effect of the cyclin D1/CDK4 complex[14]. During the regulation of cell apoptosis, Bax and Bcl-2 regulate the mitochondria-dependent pathway[21], while Fas and FasL activate the death receptor-

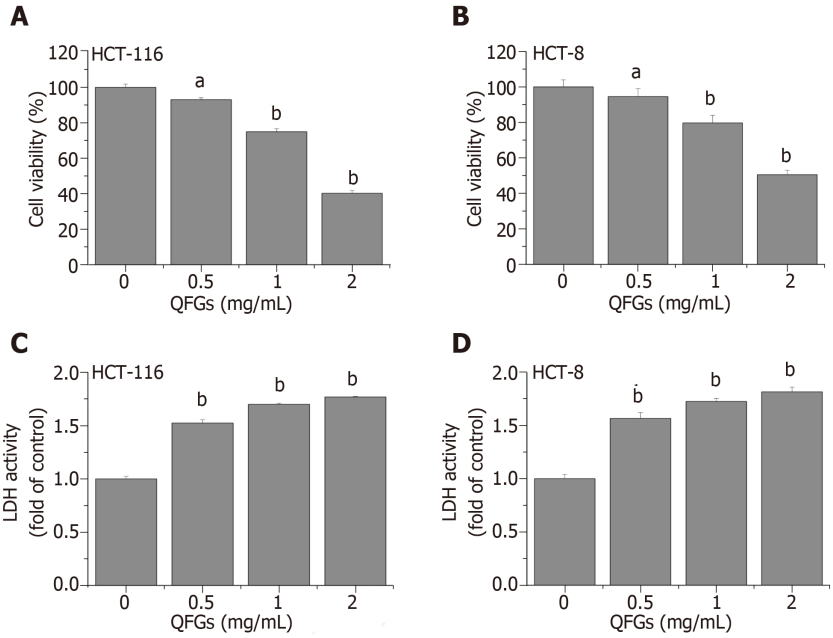


Figure 1 Effect of QFGs on the viability and cytotoxicity of HCT-116 and HCT-8 cells. A, B: Cell viability was measured by MTT assay; C, D: Cytotoxicity was measured by LDH assay. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* the control group. QFGs: Qingjie Fuzheng granules; LDH: Lactate dehydrogenase.

mediated pathway[24]. We found that QFGs can inhibit proliferation *via* arrest of the cell cycle in HCT-116 and HCT-8 cells. We used western blotting to test the protein expression levels of survivin, cyclin D1, CDK4, and p21 after the cells were treated with QFGs. As shown in Figure 5A and B, we found that QFGs downregulated the expression of survivin, cyclin D1 and CDK4, but upregulated the level of p21 in both cell types ($P < 0.05$ and 0.01, respectively). Since we found that QFGs induced apoptosis *via* the mitochondria-dependent and death receptor-mediated pathways in HCT-116 and HCT-8 cells, we used western blotting to test the protein expression levels of Bcl-2, Bax, Fas, and FasL after the cells were treated with QFGs. As shown in Figure 5C and D, we found that QFGs decreased the expression of Bcl-2 but promoted the expression of Bax, Fas, and FasL in both cell types ($P < 0.05$ and 0.01, respectively). Briefly, these findings suggest that QFGs inhibit proliferation *via* cell cycle arrest and induce apoptosis *via* the mitochondria-dependent and death receptor-mediated pathways in HCT-116 and HCT-8 cells.

QFGs suppressed the PI3K/AKT and ERK signaling pathways in HCT-116 and HCT-8 cells

Cancer occurrence and progression are highly associated with the regulation of multiple signaling pathways, including PI3K/AKT and ERK[25,26]. To further explore the potential mechanisms underlying the observed anticancer effects of QFGs, we examined the expression of major regulation factors involved in the PI3K/AKT and ERK signaling pathways. As shown in Figure 6A and B, after treatment with QFGs, the ratios of the phosphorylation expression level to the total expression level were significantly downregulated in PI3K, AKT and ERK in both cell types ($P < 0.05$ and 0.01, respectively), which suggested that the anticancer effect of QFGs on CRC cells occurs *via* suppression of the PI3K/AKT and ERK signaling pathways.

DISCUSSION

CRC is a deadly disease, primarily due to its high rate of metastasis and recurrence in patients. Multidrug combination therapy and surgical treatment are the main therapeutic methods that can significantly improve patients survival. However, there are many patients with cancer that is drug resistant and recurrent after traditional clinical treatment[27,28]. Therefore, there is a need to discover new kinds of anticancer drugs, such as herbal products. In recent years, TCM has attracted increasing attention in the field of oncology because of its relative security and long history of application[29-32]. QFGs are a four-herb TCM formula that consists of *Scutellaria barbata* D. Don, malt, *Hedyotis diffusa* Willd, and Astragalus. In the past few years, some

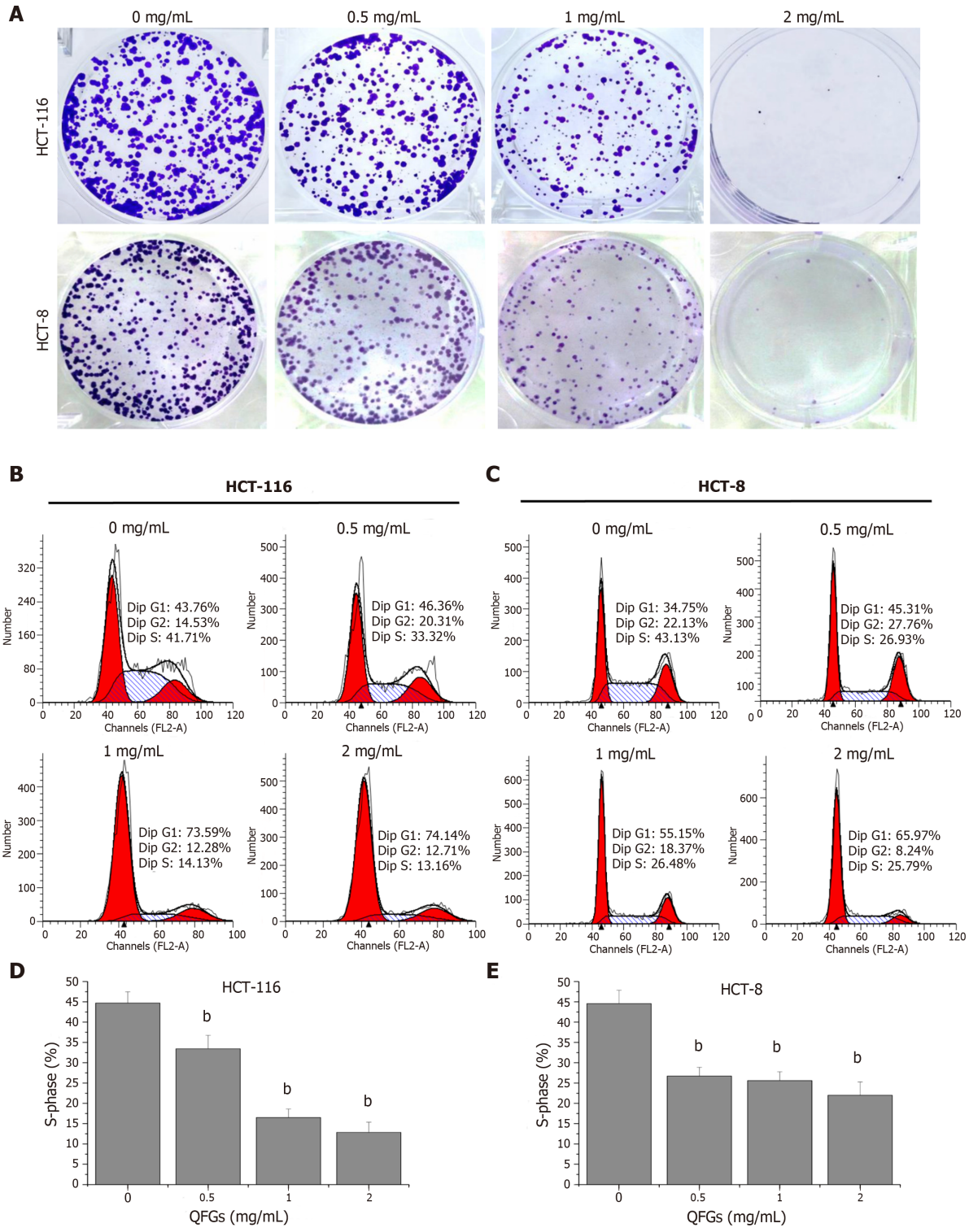
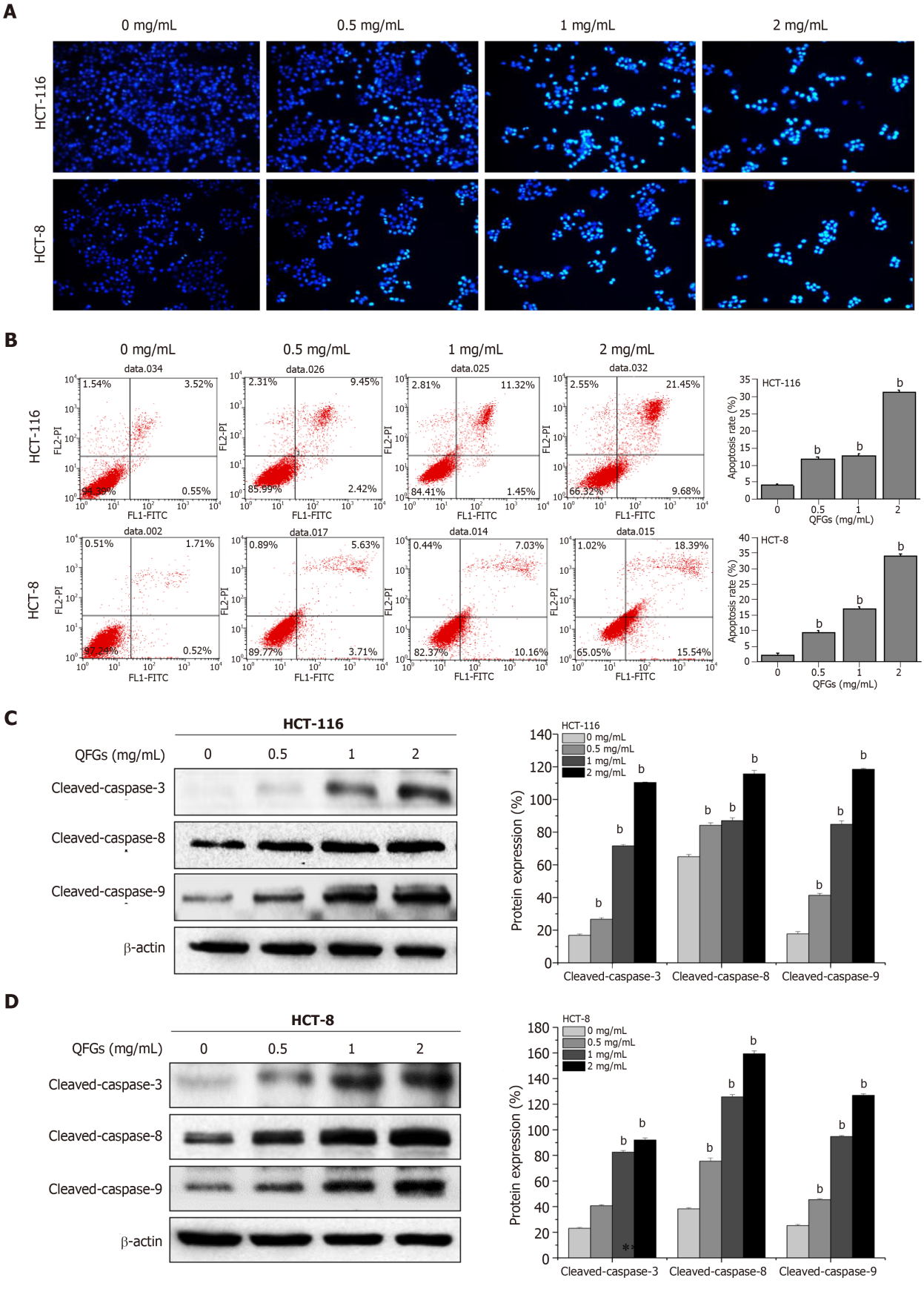


Figure 2 Effect of QFGs on the proliferation of HCT-116 and HCT-8 cells. A: Cell growth ability was measured by colony formation assays; B, C: Cell proliferation was detected by cell cycle assays with flow cytometry analysis; D, E: Quantification of FACS analysis. $^{b}P < 0.01$ *vs* the control group. QFGs: Qingjie Fuzheng granules; FACS: Fluorescence activated cell sorting.

studies have proven that *Hedyotis diffusa* Willd and *Scutellaria barbata* D. Don are capable of promoting apoptosis and inhibiting growth and angiogenesis in many types of cancer cells, including CRC[33,34]. Malt could boost the movement of Qi and improve food digestion, and Astragalus is a vital component in many TCM formulas that have been used in clinics to cure many cancer patients, reduce the incidence of complications, and improve the quality of life of cancer patients[35]. However, there are no reports on the underlying signaling pathways and mechanisms involved in QFGs anticancer activity.


A
0 mg/mL
0.5 mg/mL
1 mg/mL
2 mg/mL
HCT-116
HCT-8
B
0 mg/mL
0.5 mg/mL
1 mg/mL
2 mg/mL
HCT-116
HCT-8
FL2-PI
FL1-FITC
Apoptosis rate (%)
QFGs (mg/mL)
C
HCT-116
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β-actin
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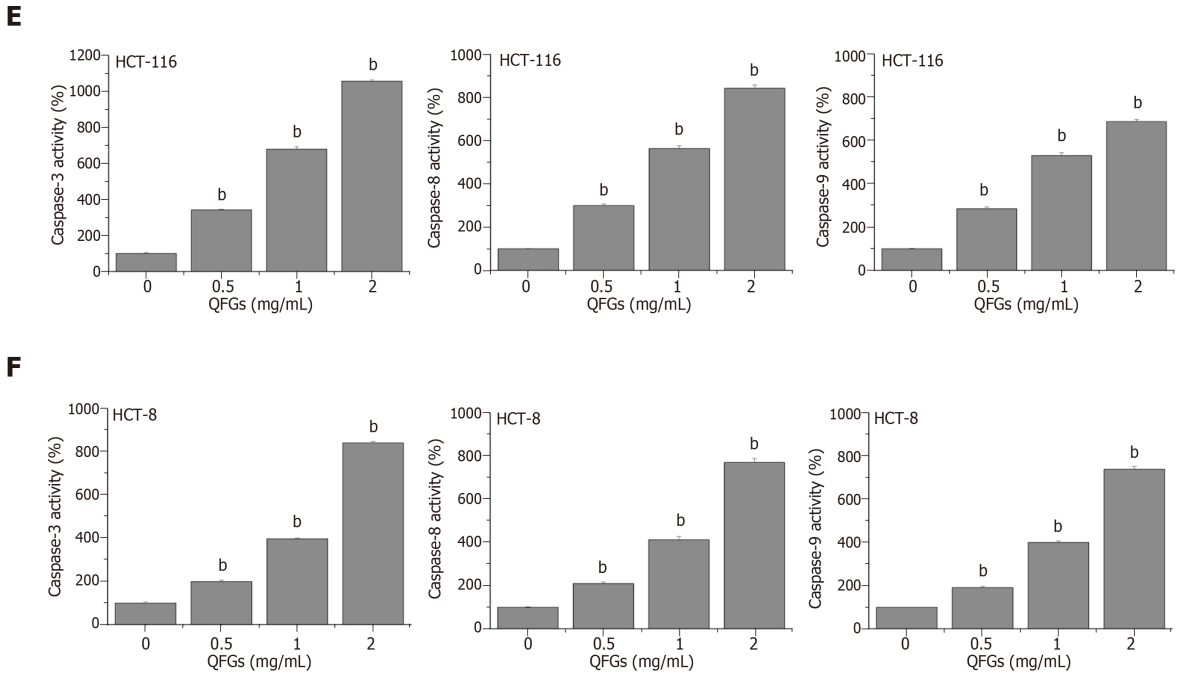

Figure 3 Effect of QFGs on HCT-116 and HCT-8 cells apoptosis. A: Cell apoptosis was detected by Hoechst 33258 staining and visualized under a fluorescent microscope (200 ×); B: Cell apoptosis was detected by Annexin V-FITC/PI staining with flow cytometry analysis, and the analysis results were quantified; C, D: The expression of cleaved caspase-3, caspase-8 and caspase-9 were determined by western blot. β-actin was used as an internal control. Each band is representative of triplicate experiments. The band densities of western blots were quantitatively analyzed; E, F: The activities of caspase-3, caspase-8 and caspase-9 were determined by using a caspase activity assay kit. $^{b}P < 0.01$ *vs* the control group. QFGs: Qingjie Fuzheng granules; PI: Propidium iodide; FITC: Fluorescein isothiocyanate.

In this study, we found that QFGs exhibited significant anticancer effects on HCT-116 and HCT-8 cells. The anticancer effect of QFGs is mainly through the inhibition of proliferation and induction of apoptosis, which are mechanisms commonly exploited in tumor therapy. As demonstrated in the current study, QFGs reduced cell viability and increased cytotoxicity in HCT-116 and HCT-8 cells in a dose-dependent manner. Furthermore, we used cell colony formation, nuclear staining, and flow cytometry assays to demonstrate that these effects in HCT-116 and HCT-8 cells resulted from the inhibition of proliferation and induction of apoptosis by QFGs.

Cell proliferation is regulated by the cell cycle, which consists of the G0, G1, S, G2 and M phases. DNA synthesis is completed in S phase, which is responsible for the initiation and completion of DNA replication[20]. Therefore, the G1/S transition is one of the two main checkpoints in the cell cycle. Using a cell cycle assay, we observed that the inhibitory effects of QFGs on HCT-116 and HCT-8 cell proliferation were associated with blocking the G1 to S phase transition. The G1/S process is highly regulated by cyclin D1, which forms complexes with CDK4[21,22]. Overexpression of the cyclin D1/CDK4 complex can enhance cell proliferation, whereas p21 can bind to this complex and inhibit its activity[24]. Therefore, the expression of CDK4, cyclin D1 and p21 indicate the proliferation state of HCT-116 and HCT-8 cells to some extent. This study proved that QFGs administration upregulates p21 protein expression while downregulating cyclin D1 and CDK4 protein expression. These results showed that QFGs inhibit HCT-116 and HCT-8 cell proliferation.

Cell apoptosis or programmed death, which is an essential process in a healthy organism, removes surplus and damaged cells[36-38]. Failure to execute the apoptosis process may lead to various diseases, such as cancer[39] and autoimmune diseases[40], whereas too much apoptosis may lead to neurodegenerative diseases[41]. There are two pathways involved in apoptosis: the mitochondria-dependent and death receptor-mediated pathways. The mitochondria-dependent pathway is initiated by caspase-9, and the death receptor pathway is initiated by caspase-8. Both pathways ultimately rely on the activation of caspase-3[17-19].

In the mitochondria-dependent apoptosis pathway, mitochondrial dysfunction directly leads to the occurrence of apoptosis and is central to the apoptotic pathway[42]. Mitochondrial outer membrane permeabilization (MOMP), an essential event in the mitochondria-mediated apoptosis pathway, causes the transfer of Cyt C and other apoptotic proteins from the mitochondria into the cytosol, which leads to caspase activation and apoptosis[43]. Caspases are the key proteins in the regulation of cell apoptosis. During mitochondria-mediated apoptosis, caspase-3 is an important

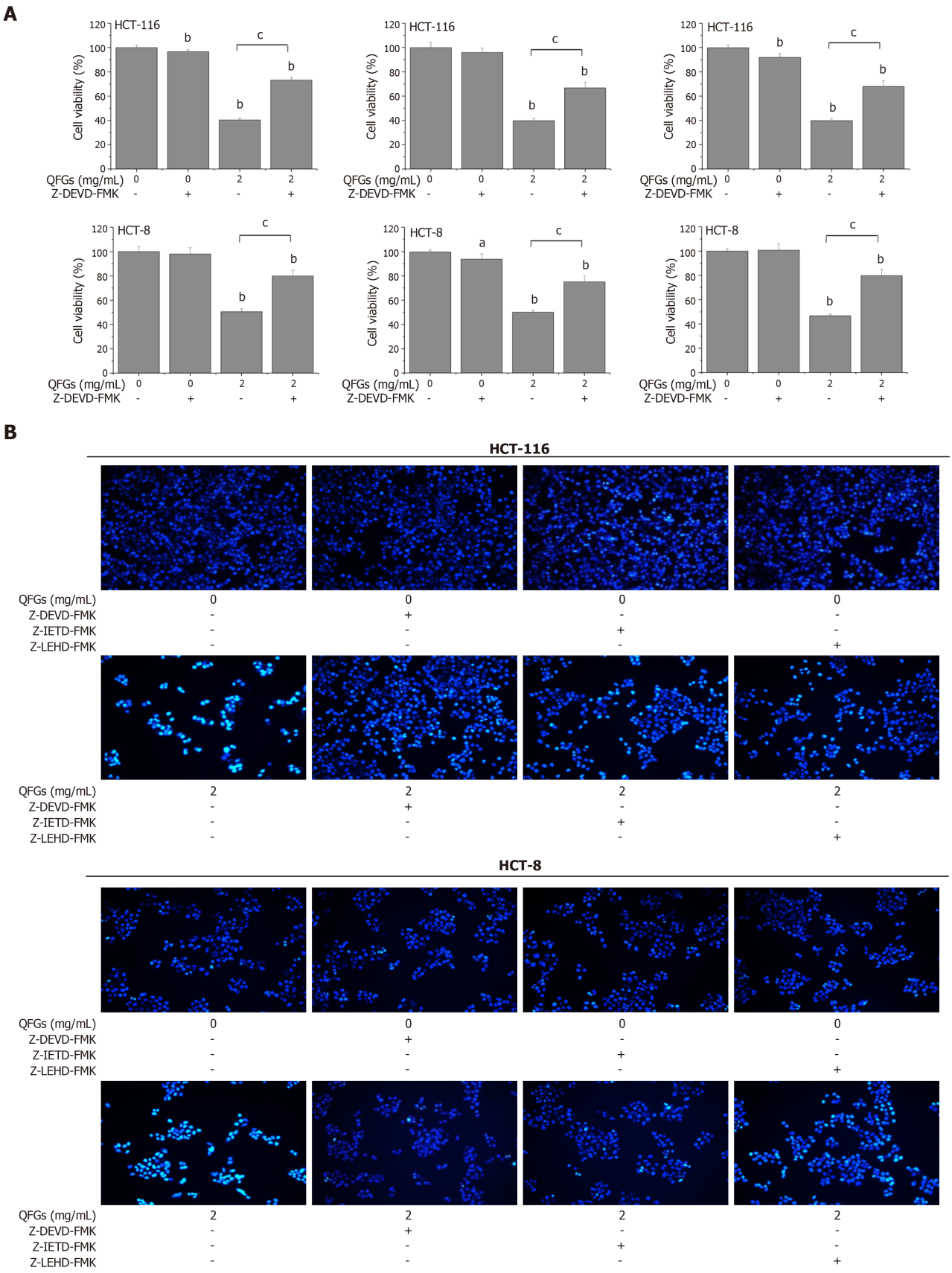

Figure 4 Effect of QFGs on the mitochondria-dependent and death receptor pathways in HCT-116 and HCT-8 cells. A: The cells were treated with or without QFGs (2 mg/mL) in combination with different caspase inhibitors (Z-DEVD-FMK, Z-IETD-FMK, Z-LEHD-FMK) for 24 h, and then cell viability was measured by MTT assay for each combination. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* the control group, $^{c}P < 0.01$ *vs* the inhibitor group; B: Cells were treated with or without QFGs (2 mg/mL) in combination with different caspase inhibitors (Z-DEVD-FMK, Z-IETD-FMK, Z-LEHD-FMK) for 24 h, and then apoptosis in the HCT-116 and HCT-8 cells was detected by Hoechst 33258 staining and visualized under a fluorescent microscope (200 ×). QFGs: Qingjie Fuzheng granules; Z-DEVD-FMK: Inhibitor of caspase-3; Z-IETD-FMK: Inhibitor of caspase-8; Z-LEHD-FMK: Inhibitor of caspase-9.

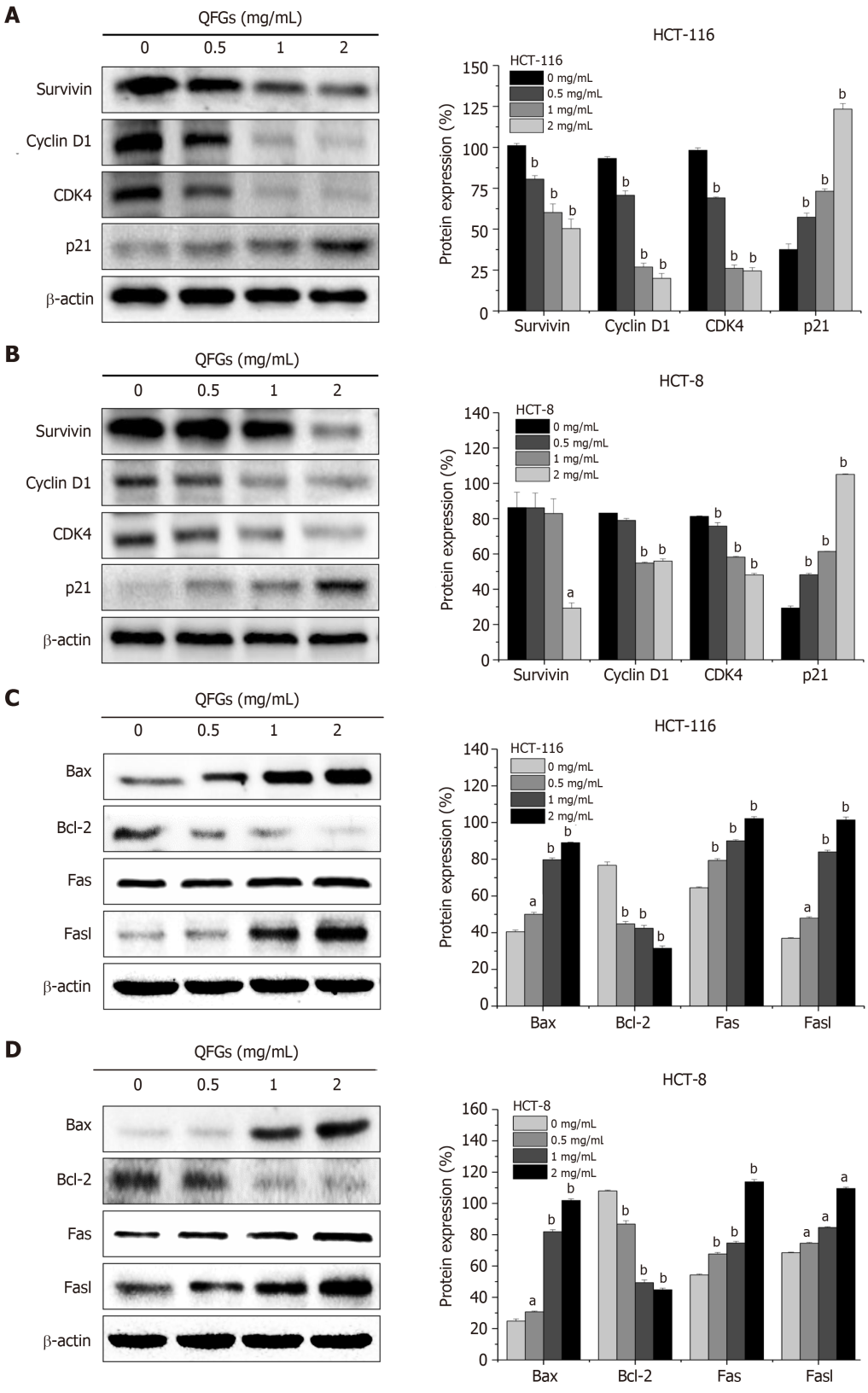

Figure 5 Effects of QFGs on the expression of survivin, cyclin D1, CDK4, p21, Bax, Bcl-2, Fas, and FasL in HCT-116 and HCT-8 cells. A, B: The expression levels of survivin, cyclin D1, CDK4, and p21 were determined by western blot. β-actin was used as an internal control. Each band is representative of triplicate experiments. The band densities of western blots were quantitatively analyzed; C, D: The expressions of Bax, Bcl-2, Fas, and FasL were determined by western blot. β-actin was used as an internal control. Each band is representative of triplicate experiments. The band densities of western blots were quantitatively analyzed. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* the control group. QFGs: Qingjie Fuzheng granules; CDK4: Cyclin-dependent kinase 4; Bcl-2: B cell leukemia/lymphoma 2; Bax: Bcl2 associated X; FasL: Fas ligand.

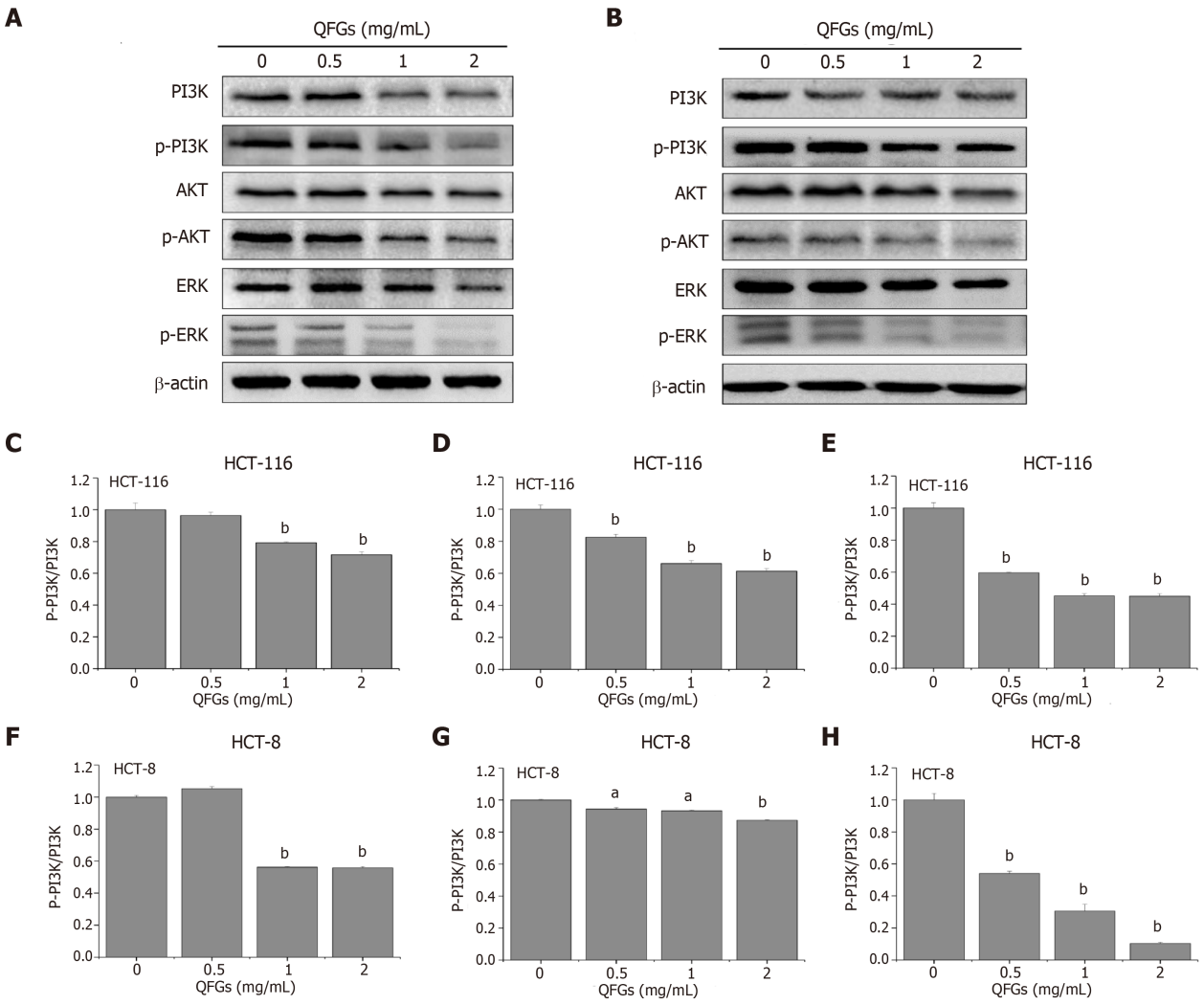

Figure 6 Effect of QFGs on the regulation of PI3K/AKT and ERK signaling pathways in HCT-116 and HCT-8 cells. A, B: The expression and phosphorylation levels of PI3K, AKT and ERK in cells were determined by western blot. β-actin was used as an internal control. Each band is representative of triplicate experiments; C-H: The ratio of phosphorylation expression level/total expression level was quantitatively analyzed. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* the control group. QFGs: Qingjie Fuzheng granules; PI3K: Phosphatidylinositol 3-kinase; pPI3K, PhosphorylatedPI3K; AKT: Protein kinase B; pAKT: PhosphorylatedAKT; ERK: Extracellular regulated protein kinases.

activator that can be cleaved by its upstream initiators, such as caspase-9. The present study showed that QFGs promoted the activation of both caspase-9 and caspase-3 in HCT-116 and HCT-8 cells. In addition, the process of HCT-116 and HCT-8 cell death induced by QFGs was followed by an increase in the cleavage of caspases-9 and caspase-3, which then promotes the molecular cascade leading to apoptosis. The Bcl-2 protein is the key regulatory protein in mitochondria-dependent apoptosis. Some studies have reported that MOMP occurs when proapoptotic Bax-like proteins form pores in the mitochondria; however, the effect of anti-apoptotic Bcl-2-like members on MOMP is opposite to that of proapoptotic Bax-like proteins on MOMP. Therefore, the ratio of Bax to Bcl-2 is the key to determining cell survival [44,45]. Our study proved that QFGs administration upregulated Bax protein expression and downregulated Bcl-2 protein expression. These results showed that QFGs induce HCT-116 and HCT-8 cell apoptosis *via* the mitochondria-dependent pathway.

During the death receptor apoptosis pathway, caspase-3 is also the ultimate activator of apoptosis. Caspase-3 is cleaved by its downstream initiators, such as caspase-8. In this study, we discovered that both caspase-8 and caspase-3 can be cleaved by QFGs in HCT-116 and HCT-8 cells. In addition, as noted earlier, HCT-116 and HCT-8 cell death induced by QFGs was followed by increased cleavage of caspase-8 and caspase-3, which accelerates apoptosis. In this pathway, death signals are transmitted *via* cell surface receptors that communicate with the FasL/Fas signaling pathway, which is part of the death receptor pathway. After binding to FasL, Fas trimerizes and interacts with Fas-associated protein with a death domain, which contributes to the cleavage of caspase-8 and caspase-10 and leads to activation

of downstream effector caspases, including caspase-3, caspase-6 and caspase-7, ultimately causing apoptosis[24]. In this study, we demonstrated that QFGs treatment upregulated FasL and Fas protein expression. These results showed that QFGs induce HCT-116 and HCT-8 cell apoptosis through the extrinsic apoptosis pathway.

To determine if the two classic apoptosis pathways were both involved in this study, we added caspase-3/-8/-9 inhibitors and performed the MTT and Hoechst 33258 staining assays to test cell viability and cell apoptosis once again. We found that all three inhibitors markedly inhibited the inhibitory effect of QFGs on cell viability in CRC cells, and inhibited the apoptosis induced by QFGs. This result verified that QFGs induced apoptosis *via* the mitochondria-dependent and death receptor apoptosis pathways in HCT-116 and HCT-8 cells, which directly revealed the multi-target inhibitory effects of QFGs on CRC cells.

The pathogenic mechanisms underlying the development of cancer, including CRC, are heterogeneous and regulated by multiple signaling pathways, including PI3K/AKT and ERK[25,26]. Previous studies have reported that the PI3K/AKT and ERK signaling pathways regulate cell growth, apoptosis and metastasis[46]. As one of the important intracellular signal transduction pathways, PI3K/AKT signaling has been reported to play important roles in cell survival, apoptosis and metastasis [47]. In previous studies, activated AKT existed in CRC tumors, which has been shown to be a poor prognostic factor for CRC patients[48]. Overexpression of downstream factors of AKT may result in the activation of the PI3K signaling pathway[46]. ERK signaling is also an important pathway that highly regulates cell proliferation and apoptosis. ERK is a mitogen-activated protein kinase (MAPK), which can be activated by MAPK kinase kinase (*e.g.*, Raf), MAPK kinase (*e.g.*, MEK), and MAPK (*e.g.*, ERK)[49]. Activation of the ERK pathway regulates the expression of various genes and proteins that mediate cell proliferation and apoptosis. The present study demonstrated that QFGs suppressed the activations of PI3K, AKT and ERK, which showed that the anticancer effect of QFGs acts on CRC cells *via* the PI3K/AKT and ERK signaling pathways.

ARTICLE HIGHLIGHTS

Research background

Colorectal cancer (CRC) is a major public health problem, representing the third cause of cancer deaths worldwide. Surgery and adjuvant chemotherapy are the main treatment for CRC. However, 40-50% of patients still die due to recurrence, metastases and drug resistance. In addition, severe side effects caused by chemotherapy agents lead to the deterioration of patient quality-of-life and therapeutic application. Therefore, the search for novel therapies has attracted worldwide attention. Qingjie Fuzheng granules (QFGs) is a traditional Chinese medicine formula with properties of anti-inflammation, antioxidative, antibacterial, immunity enhancement, and digestion promotion. QFGs has been widely used and found to be clinically effective in various cancer treatments, including CRC, and has few side effects. However, the precise mechanisms and molecular signaling pathways involved in the activity of QFGs' anticancer effects have not been reported in the literature. In this study, we hypothesized that QFGs can inhibit the growth of CRC cells, and that its mechanism is closely related to one or more intracellular signal transduction pathways.

Research motivation

To better understand the mechanism underlying the potential anti-cancer effect of QFGs on the human CRC cell variants HCT-116 and HCT-8.

Research objectives

To elucidate the effect of QFGs on the biological function of CRC cells, and to investigate this biological function to explore the exact mechanism of QFGs effects on CRC cells.

Research methods

First, cell viability and cytotoxicity were measured by performing MTT and LDH assays. We evaluated the role of QFGs in cell proliferation and apoptosis by assessing colony formation using Hoechst 33258. Second, cell cycle and apoptosis levels were measured by fluorescence-activated cell sorting. The expression levels of survivin, cyclin D1, CDK4, p21, Bax, Bcl-2, Fas, FasL, and cleaved-caspase-3/-8/-9 were measured by performing western blotting and caspase activity assays. Furthermore, inhibitors of caspase-3/-8/-9 were also used to elucidate the exact apoptosis pathway induced by QFGs in cancer cells. Finally, activation of the PI3K/AKT and ERK signaling pathways was examined using the western blot assay to investigate the possible mechanism.

Research results

MTT and LDH assays revealed that after 0.5-2.0 mg/mL of QFGs treatment, cell viability was reduced by (6.90% ± 1.03%)–(59.70% ± 1.51%) (HCT-116; $P < 0.05$) and (5.56% ± 4.52%)–(49.44% ± 2.47%) (HCT-8; $P < 0.05$). Cytotoxicity was increased from 0.52 ± 0.023 to 0.77±0.002 (HCT-116; P

< 0.01) and from 0.56 ± 0.054 to 0.81 ± 0.044 (HCT-8; P < 0.01) compared with non-QFGs treatment groups. Additionally, colony formation and Hoechst 33258 staining assays showed that QFGs inhibited proliferation and induced apoptosis in CRC cells. QFGs also increased the expression levels of Bax, Fas, and FasL, decreased the level of Bcl-2, and stimulated the activation of caspase-3/-8/-9, which were revealed by western blot and caspase activity assays. In contrast, upon adding the three caspase inhibitors, the suppression effect of QFGs on cell viability and apoptosis were markedly inhibited. Moreover, QFGs suppressed the phosphorylation levels of PI3K, AKT and ERK.

Research conclusions

These results demonstrated that QFGs inhibit CRC cell proliferation and induce apoptosis by suppressing the PI3K/AKT and ERK signaling pathways. This indicated that QFGs are a potential new therapeutic treatment for CRC and other cancers.

Research perspectives

Traditional Chinese Medicine (TCM) is important for the treatment of many cancers and has a long history of clinical use. If the effects and mechanisms of TCM are further elucidated, it may provide a more effective treatment for many cancer types.

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ORIGINAL ARTICLE

Retrospective Study

Surgical complications after different therapeutic approaches for locally advanced rectal cancer

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Abstract

BACKGROUND

Preoperative radiochemotherapy is widely used in locally advanced rectal cancer. It can improve local control of rectal cancer. However, some researchers believe it increases the incidence of surgical complications. They doubt its safety. Patients with locally advanced rectal cancer receive three different treatments in our hospital, including long-course radiochemotherapy, short-course radiotherapy, and surgery directly. We can compare their differences in postoperative complications.

AIM

To investigate surgical complications caused by different preoperative radiotherapy regimens.

METHODS

We retrospectively analyzed 1197 patients admitted between 2008 and 2010 with locally advanced rectal cancer. Three hundred and forty-six patients were treated with preoperative long-course radiochemotherapy (25 × 2 Gy) followed by total mesorectal excision (TME) 6–8 wk later, and 259 patients received short-course radiotherapy (10 × 3 Gy) and subsequently TME 7–10 d later. The remaining 592 patients underwent TME alone without neoadjuvant therapy. According to Clavien–Dindo classification, surgical complications were evaluated for up to 30 d after discharge from hospital.

RESULTS

There were no deaths in 30 d in all groups after treatment. The major

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complications were anastomotic leakage and perineal wound complications. The results suggested that both long-course [odds ratio (OR) = 3.624, 95% confidence interval (CI): 1.689–7.775, $P = 0.001$] and short-course (OR = 5.150, 95%CI: 1.828–14.515, $P = 0.002$) radiotherapy were associated with anastomotic leakage. Temporary ileostomy was a protective factor for anastomotic leakage (OR = 6.211, 95%CI: 2.525–15.385, $P < 0.001$). The severity of anastomotic leakage did not increase in patients following preoperative radiotherapy ($P = 0.411$). Compared with TME alone, short-course radiotherapy was associated with an increase in perineal wound complications (OR = 5.565, 95%CI: 2.203–14.057, $P < 0.001$), but long-course radiotherapy seemed safe regarding this complication (OR = 1.692, 95%CI: 0.651–4.394, $P = 0.280$). Although the severity of perineal wound complications increased in patients following short-course radiotherapy ($P < 0.001$), additional intervention was not necessary.

CONCLUSION
Radiotherapy increased the incidence but not severity of anastomotic leakage. Short-course radiotherapy was also accompanied with perineal wound complications, but intervention appeared unnecessary to ameliorate the complications.

Key words: Rectal cancer; Radiotherapy; Surgical complications; Total mesorectal excision; Anastomotic leakage

Core tip: Preoperative radiotherapy is a promising treatment for rectal cancer. Our aim is to investigate surgical complications caused by radiotherapy. Both long-course and short-course radiotherapy increased the incidence of anastomotic leakage but did not affect the severity. Additional ileostomy was an effective method to reduce the risk of anastomotic leakage. Short-course radiotherapy was accompanied with increased incidence of perineal wound complications, but intervention appeared unnecessary to ameliorate the complications.

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INTRODUCTION

Patients with locally advanced rectal cancer were recommended to receive neoadjuvant radiochemotherapy, especially for those with positive circumferential resection margin or extensive nodal involvement. It can improve local control for these patients. The rate of local recurrence has decreased significantly as a result of neoadjuvant radiochemotherapy. Some researchers believe that preoperative radiotherapy can improve survival of patients with resectable rectal cancer. It is suggested that patients who are sensitive to radiotherapy can achieve better prognosis. Approximately 15% of patients can achieve complete response after long-course neoadjuvant radiochemotherapy[1]. Different protocols for short-course radiotherapy that consists of 30 Gy in 10 fractions are recommended by the Chinese Anti-Cancer Association[2]. The biological equivalent dose of the short-course radiotherapy is similar to the commonly used regimen (5 × 5 Gy). Although the tumor regression is not as good as with long-course radiochemotherapy, with < 5% complete response rate, the advantages are no surgical delay, reduced toxicity from capecitabine, and avoidance of overtreatment of non-responders. However, some surgeons believe that preoperative radiochemotherapy increases surgical complications. Anastomotic leakage is thought to be associated with malnutrition resulting from radiotherapy[3]. Perineal wound complications after abdominoperineal resection (APR) are also considered to be associated with tissue edema and infection caused by radiotherapy. In addition, toxicity of radiochemotherapy may decrease patients' tolerance to surgery.

The aim of this study was to evaluate surgical complications of patients with locally advanced rectal cancer following different neoadjuvant therapy and radical surgery. We compared the incidence and severity of surgical complications at 30 d after surgery in different groups and the contribution of neoadjuvant therapy to surgical complications.

MATERIALS AND METHODS

Patients

We performed a retrospective consecutive study of 1197 patients with mid-to-low rectal cancer (≤ 10 cm from anal verge) who received low anterior resection and APR at the Peking University Cancer Hospital between 2008 and 2010. Among them, 346 patients were treated with long-course chemoradiotherapy, and 259 received short-course radiotherapy. Radical resection was performed in all patients. The remaining 592 patients received total mesorectal excision (TME) immediately after rectal cancer was diagnosed. Surgical complications were evaluated for up to 30 d after discharge from hospital according to Clavien–Dindo classification. The median duration of admission for patients who underwent resection was 19 (range 5–81) d. Among them, 197 patients (16.3%) were hospitalized for > 30 d.

Radiotherapy

Two different neoadjuvant radiotherapy regimens were applied. Three hundred and forty-six patients received long-course preoperative radiochemotherapy that consisted of 50 Gy in 25 fractions with capecitabine (825 mg/m^2, twice daily) as radiosensitizer. The other 259 patients were treated with short-course radiotherapy that consisted of 30 Gy in 10 fractions. Its biological equivalent dose was 36 Gy, which was close to the dose of 5 × 5 Gy radiation (37.5 Gy).

Surgery

TME was the standard approach for surgical treatment of rectal cancer. All patients underwent laparotomy at Beijing Cancer Hospital at 6–8 wk after long-course radiochemotherapy or 7–10 d after short-course radiotherapy. Low anterior resection (LAR) was performed in 894 patients. Temporary ileostomy was performed based on the pathological conditions during the operation. APR was performed in 303 patients.

Surgical complications

Surgical complications were evaluated using predetermined conditions of common complications (Table 1). The main complications are anastomotic leakage and perineal wound complications. The definition of anastomotic leakage was different from those in the literature. It was confirmed by detection of fluid collection through the drainage tubes. Digital rectal examinations were used to evaluate the size of the leakage. Computed tomography was not routinely performed unless puncture drainage or surgical reintervention was needed. The severity of these complications was evaluated by Clavien–Dindo classification (Table 2).

Statistical analysis

The association between neoadjuvant radiotherapy and surgical complications was analyzed using two-sided χ^2 or Fisher's exact test. The two key complications, anastomotic leakage and perineal wound complications, were also evaluated. The clinical variables included general information about the patients and tumor characteristics, as well treatment-related variables such as diverting ileostomy. Logistic regression was performed to investigate the independent factors associated with anastomotic leakage and perineal would complications. $P < 0.05$ was considered as statistically significant.

RESULTS

Groups and patient characteristics

A total of 1197 patients with locally advanced rectal cancer who received LAR and APR were analyzed. They all underwent laparotomy. The patients were divided into three groups according to different preoperative therapy (Figure 1). Group 1: 346 patients treated with preoperative long-course chemoradiotherapy followed by TME 6–8 wk after. Group 2: 259 patients were treated with short-course radiotherapy (10 × 3 Gy) followed by TME 7–10 d after. Group 3: 592 patients received radical surgery only. Patient and tumor characteristics are summarized in Table 3. The median

Table 1 Definition of postoperative surgical complications (during admission and 30 d thereafter)

	Definition
Anastomotic leakage	Any gas or feces collection around the anastomosis after low anterior resection in the drainage tubes; clinical suspicion confirmed by surgery
Perineal wound complications	Perineal wound dehiscence and wound necrosis after abdominoperineal resection resulting from infection
Ileus	Absence of bowel sounds or defecation after 5 d following surgery
Bleeding	Gastrointestinal or abdominal hemorrhage, decrease in hemoglobin level directly after surgery treated conservatively with blood transfusion or by reintervention
Intra-abdominal abscess	Any intra-abdominal fluid collection unrelated to the anastomosis or perineal wound
Abdominal wound complications	Fascial dehiscence, superficial wound infection
Urological complications	Ureter leakage, urinary incontinence, ureter stenosis
Intestinal necrosis	Caused by bowel ischemia
Gastrointestinal perforation	Intestinal contents discharge from abdominal cavity; clinical suspicion confirmed by surgery
Intravenous line infection	Fever, chills and increase in leukocyte count, excluding other infections; the symptoms disappear after removing the intravenous line
Stoma complications	Stoma necrosis, stoma infection, parastomal hernia
General complications	Cardiovascular, pulmonary, neurological events

duration of admission for patients who underwent resection was 19 (range 5–81) d. One hundred and ninety-seven patients (16.3%) stayed in the hospital for > 30 d.

Treatment-related postoperative complications

Eight hundred and ninety-four patients underwent LAR, and 303 patients received APR. There were no deaths within 30 d after surgery. Forty-three patients required surgical reintervention. We analyzed 12 different complications, including anastomotic leakage, perineal wound complications, ileus, bleeding, intra-abdominal abscess, abdominal wound complications, urological complications, intestinal necrosis, gastrointestinal perforation, intravenous line infection, stoma complications, and general complications. Anastomotic leakage and perineal wound complications were the two major complications after resection. The severity of postoperative complications is summarized in Table 4. There were no significant differences in the grade of treatment-related complications except for perineal wound complications. Higher grade of perineal wound complication was observed in patients following short-course radiotherapy.

In 894 patients who received LAR, anastomotic leakage was the most obvious complication. Anastomotic leakage developed in 48 (5.4%) patients. Nineteen (2.1%) patients who required surgical reintervention were classified as Grade 3b according to the Clavien–Dindo classification. Our data suggested that preoperative radiotherapy (P = 0.001) and diverting ileostomy (P < 0.001) were significant independent factors (Table 5). Both long-course [odds ratio (OR) = 3.624, 95% confidence interval (CI): 1.689–7.775, P = 0.001] and short-course (OR = 5.150, 95%CI: 1.828–14.515, P = 0.002) neoadjuvant radiotherapy increased the incidence of anastomotic leakage (6.78%, 5.96%, and 4.54% in Groups 1, 2, and 3, respectively), but neither was associated with the severity of the complication (P = 0.411) (Table 4). Temporary diverting ileostomy was a protective factor to reduce the incidence of anastomotic leakage (OR = 6.211, 95%CI: 2.525–15.385, P < 0.001). The majority of patients with neoadjuvant radiotherapy underwent additional surgery of temporary ileostomy, especially in those with short-course radiotherapy (69.4%, 83.1%, and 7.4% in Groups 1, 2, and 3, respectively, P < 0.001).

Three hundred and three patients received APR. More than 16.5% of patients suffered from perineal wound complications. The incidence of perineal wound complications in the three groups was 11.8%, 26.8%, and 9.4%, respectively. Short-course chemoradiotherapy was closely associated with perineal wound complications (OR = 5.565, 95%CI: 2.203–14.057, P < 0.001). In contrast, long-course radiochemotherapy did not significantly influence development of perineal wound complications (OR = 1.692, 95%CI: 0.651–4.394, P = 0.280) (Table 6). The grade of these complications differed significantly among the three groups (P < 0.001) (Table 4). Patients receiving short-course radiotherapy had higher-grade perineal wound complications. However, there were no Grade 3b perineal wound complications, and

Table 2 Clavien–Dindo classification of surgical complications

Grade	Definition
I	Any deviation from the normal postoperative course without the need for pharmacological treatment or surgical, endoscopic and radiologic interventions. Allowed therapeutic regimens are drugs including antiemetics, antipyretics, analgesics and diuretics, and electrolytes and physiotherapy. This grade also includes wound infections opened at the bedside.
II	Requiring pharmacological treatment with drugs other than those allowed for grade I complications. Blood transfusions and total parenteral nutrition are also included.
III	Requiring surgical, endoscopic or radiological intervention
IIIa	Intervention not under general anesthesia
IIIb	Intervention under general anesthesia
IV	Life-threatening complication (including CNS complications)[1] requiring IC/ICU management
IVa	Single organ dysfunction (including dialysis)
IVb	Multiple organ dysfunction
V	Death as a result of complications

[1]Brain hemorrhage, ischemic stroke, or subarachnoid bleeding, but excluding transient ischemic attacks. Adapted from Clavien–Dindo classification. CNS: Central nervous system; IC: Intermediate care; ICU: Intensive care unit.

none of these patients required surgical reintervention.

Reintervention

Among all the studied patients, only 43 with Grade 3b complications required reintervention. The reasons included anastomotic leakage, ileus, bleeding, intra-abdominal abscess, abdominal wound complications, urological complications, and intestinal necrosis. Some patients with anastomotic leakage required reintervention. The reintervention rate for anastomotic leakage repair in in all three groups did not differ significantly (37.5%, 66.7%, and 30.4% in Groups 1, 2, and 3, respectively, P = 0.411), indicating that neither long-course nor short-course radiotherapy increased the need for reintervention. The increase in perineal wound complications caused by short-course radiotherapy was mild. None of the patients with perineal wound complications required reintervention.

DISCUSSION

Neoadjuvant radiochemotherapy has become important to reduce local recurrence of locally advanced rectal cancer. In China, an increasing number of patients have been recommended to receive radiotherapy before surgery, but an increase in postoperative complications if patients receive preoperative radiation has been a major concern[4]. The present study compared the major postoperative complications associated with long-course and short-course radiotherapy followed by TME in a large series of patients with locally advanced rectal cancer. Compared to those without radiotherapy, the increase in surgical complications caused by two different preoperative radiotherapy regimens was acceptable, and postoperative mortality did not increase. No patients died within 30 d after surgery, although preoperative radiotherapy might have been associated with anastomotic leakage and perineal wound complications. Temporary ileostomy prevented the occurrence and severity of anastomotic leakage. The grade of surgical complications did not differ significantly, except for perineal wounds, which did not always require surgical reintervention.

Several studies have investigated whether preoperative radiotherapy increases surgical complications[5]. The conclusions were not in agreement. Most of these studies have suggested that preoperative radiotherapy does not increase postoperative morbidity[6]. However, the complications in patients with neoadjuvant radiotherapy seem to be more severe, as demonstrated by the need for more surgical reintervention to treat the complications[7]. Our study indicated that long-course and short-course chemoradiotherapy were not associated with increased incidence or grade of complications. Conservative measures do not have any benefit after radiotherapy[8]. Reintervention is more often used for patients who receive radiotherapy if anastomotic leakage cannot be healed. This was one of the major setbacks of

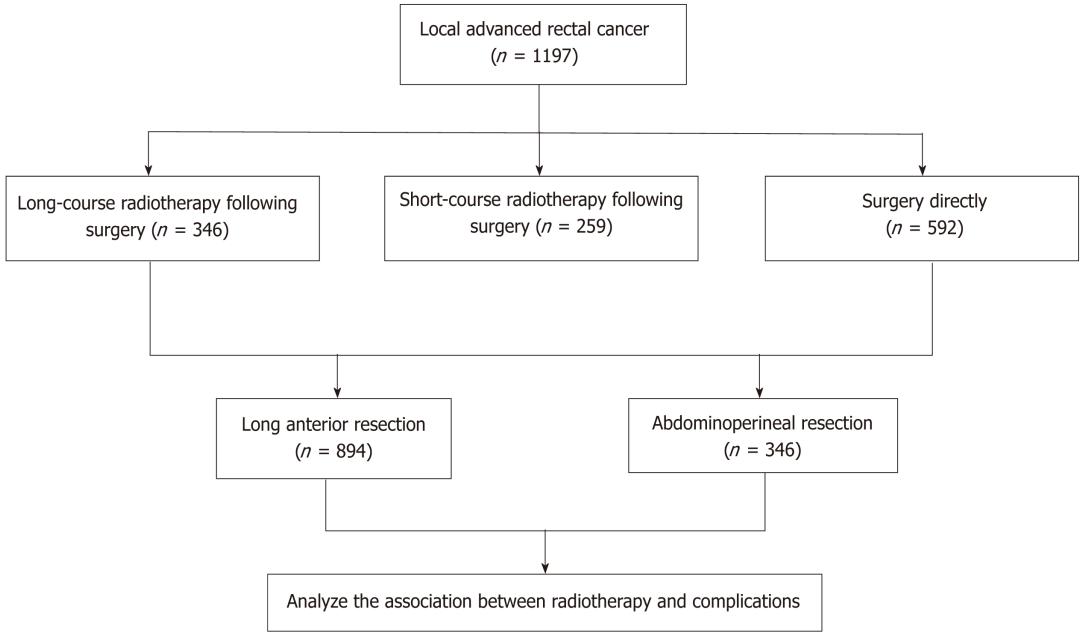

Figure 1 Flow diagram of treatment.

radiotherapy.

Anastomotic leakage is the most serious surgical complication after LAR for rectal cancer. It occurs in 3.5%–25.0% of patients after surgery[9,10]. The rates reported varied according to different definitions being used. In some studies, anastomotic leakage was diagnosed by computed tomography, magnetic resonance imaging, or radiography. The incidence rates were usually higher by imaging diagnosis than clinical observation. In this study, we defined leakage by the presence of gas or feces around the anastomosis in the drainage tubes. Radiology was used only when surgical reintervention was required. There were many risk factors believed to be associated with anastomotic leakage, such as male gender, lower location of the tumor, and preoperative radiotherapy[11,12]. Neoadjuvant radiotherapy has been implicated as a causative factor for the increased rate of anastomotic leakage[13]. It might be due to the local effect of radiation, and subsequently increased technical difficulty during the operation. Radiotherapy may also decrease the oxygen supply to the local tissue around the anastomosis. It can slow down the healing process and cause leakage. In our study, the rate for all these patients was 5.4%. It was a low incidence and within the acceptable range compared with other complications.

It is agreed that preoperative radiotherapy is associated with an increase in anastomotic leakage. Patients with radiotherapy may need a longer time for healing of leakage. As a result, a temporary defunctioning ileostomy was constructed during rectal surgery for patients who underwent preoperative radiotherapy, and it was reversed after 6 mo[14]. It is believed that ileostomy can reduce anastomotic leakage[15]. In the present study, more patients with radiotherapy had a defunctioning ileostomy than those who underwent surgery alone. Defunctioning ileostomy also decreases the grade of leakage as the feces are diverted[16]. This may be an effective approach to avoid surgical reintervention caused by anastomotic leakage.

Perineal wound complications are commonly seen after APR. Previously reported rates varied between 5.9% and 31.0%[17,18]. Most perineal wound complications, such as wound gaping, are mild and do not require a prolonged stay in hospital or surgical reintervention. However, severe wound complications can impair quality of life[19]. For example, patients with open wounds are usually accompanied with pain and movement limitation. They might also delay subsequent adjuvant chemotherapy, which may result in worse prognosis. Previous studies have shown that the rate of perineal wound complications increases in patients who receive preoperative radiotherapy[4,20]. Our study also showed a significant increase in perineal wound complications after short-course radiochemotherapy compared with patients who received long-course raidochemotherapy and those who did not receive neoadjuvant treatment. In addition, the grade of the complications was also higher in the short-course radiotherapy group. The grade of these complications was always 3a. However, no reintervention was required to manage these complications.

Table 3 Patient and treatment characteristics

Characteristics	No. of patients
Age in yr	59 (21–88)
Sex ratio, male: female	721: 485
Zubrod-ECOG-WHO	
0	1060
1	106
2	40
3	0
4	0
5	0
Preoperative treatment	
25 × 2 Gy with capecitabine	346
10 × 3 Gy	259
None	592
Distance from anal verge	
≤ 5 cm	817
5–10 cm	389
Surgery	
LAR	894
APR	303
Hartmann procedure	27
No resection	7
Diverting stoma after LAR	
Yes	313
No	581
Pathological TNM classification	
pT0	70
pT1	59
pT2	326
pT3	661
pT4	81
pN0	687
pN1	284
pN2	226

ECOG-WHO: Eastern Cooperative Oncology Group-World Health Organization; LAR: Low anterior resection; APR: Abdominoperineal resection.

Although our study had a large patient cohort, it was limited by its retrospective nature. It is difficult to fully evaluate late complications for > 30 d after hospital discharge, and the incidence might have been underestimated.

The use of preoperative radiochemotherapy for rectal cancer has been debated for decades, including indications, methods, TRG, and so on. Preoperative chemoradiotherapy may be affected by several factors, such as carcinoembryonic antigen and histological regression score. Acellular mucin pools are also thought to be a useful predictor for complete response in several studies[21,22], but it is controversial. The association between postoperative complications and mucin pools is worth study. From 2008-2010, which is the recruitment period, we had not begun to detect routinely regression rate and mucin pool in our hospital. Therefore, these data were not collected for this study. It is one of the limitations of our study.

Since the sample includes more 1000 patients, we can compare the local control, survival, and quality of life among different groups. We can compare differences of clinical effect between different preoperative therapies, and additional studies are necessary in the future.

In conclusion, preoperative radiotherapy was associated with two major surgical complications: anastomotic leakage and perineal wound complications. There were no significant differences in other complications. Both long-course and short-course

Table 4 Postoperative complications (events during admission and 30 d thereafter)

Complications	Treatment group	No. of patients	Total	Grade 1	Grade 2	Grade 3a	Grade 3b	Grade 4a	Grade 4b	Grade 5	*P* value
Anastomotic leakage (LAR)	1	236	16		10		6				0.411
	2	151	9		3		6				
	3	507	23		16		7				
Perineal wound complications (APR)	1	110	13	9	3	1					< 0.001
	2	108	29	14	13	2					
	3	85	8	4	3	1					
Ileus	1	346	11	2	5		4				0.069
	2	259	14		13		1				
	3	592	14	2	12						
Bleeding	1	346	10	1	8		1				0.485
	2	259	10	2	7						
	3	592	14	2	10		2				
Intra-abdominal abscess	1	346	12	2	5	4	1				0.932
	2	259	10		5	2	3				
	3	592	20	3	8	6	3				
Abdominal wound complications	1	346	10	7	2		1				0.474
	2	259	10	6	4						
	3	592	14	11	2		1				
Urological complications	1	346	17	10	4		3				0.154
	2	259	16	5	8		3				
	3	592	20	12	6		2				
Intestinal necrosis	1	346	2				2				0.689
	2	259	3		2		1				
	3	592	4				4				
Gastrointestinal perforation	1	346	0								1.000
	2	259	0								
	3	592	0								
Intravenous line infection	1	346	8		8						0.641
	2	259	8		8						
	3	592	12		12						
Stoma complications	1	269	11	4	7						0.702
	2	228	14	3	11						
	3	119	5	2	3						
General complications	1	346	36	21	15						0.520
	2	259	34	17	17						
	3	592	54	28	25	1					

Group 1: Patients received 25 × 2 Gy radiation with capecitabine; Group 2: Patients received 10 × 3 Gy radiation; Group 3: Patients did not receive radiation; LAR: Low anterior resection; APR: Abdominoperineal resection.

radiochemotherapy increased the incidence of anastomotic leakage, but the grade remained close to that in patients treated with surgery alone. A temporary defunctioning ileostomy seemed to be an effective method to reduce the risk of anastomotic leakage for patients who received radiotherapy. Short-course radiochemotherapy increased the incidence and grade of perineal wound complications. Reintervention may not be necessary to ameliorate the perineal wound complications as the damage is usually low grade.

Table 5 Logistic regression analysis of anastomotic leakage

Characteristics	Anastomotic leakage			
	χ^2	OR	95%CI of Exp(B)	*P* value
Age	1.748	1.542	0.812–2.928	0.186
Sex	1.081	1.410	0.738–2.695	0.299
Distance from anal verge	0.508	1.276	0.653–2.496	0.476
Pathological T stage	13.089	2.620	1.555–4.415	< 0.001
Pathological N stage	1.402	0.768	0.496–1.189	0.236
Preoperative radiotherapy	14.029			0.001
Long course CRT/surgery directly	10.931	3.624	1.689–7.775	0.001
short course RT/surgery directly	9.614	5.150	1.828–14.515	0.002
diverting stoma	15.804	6.211	2.525–15.385	< 0.001

OR: Odds ratio; CRT: Chemoradiotherapy; RT: Radiotherapy.

Table 6 Logistic regression analysis of perineal wound complications

Characteristics	Perineal wound complications			
	χ^2	OR	95%CI of Exp(B)	*P* value
Age	1.576	1.508	0.794–2.865	0.209
Sex	1.542	1.513	0.787–2.907	0.214
Distance from anal verge	1.045	1.779	0.590–5.376	0.307
Pathological T stage	0.391	1.121	0.784–1.602	0.532
Pathological N stage	0.791	1.227	0.782–1.927	0.374
Preoperative radiotherapy	16.757			<0.001
long course CRT/surgery directly	1.166	1.692	0.651–4.394	0.280
short course RT/surgery directly	13.184	5.565	2.203–14.057	<0.001

OR: Odds ratio; CRT: Chemoradiotherapy; RT: Radiotherapy.

ARTICLE HIGHLIGHTS

Research background

Preoperative radiochemotherapy can improve local control of rectal cancer. However, some researchers believe it increases the incidence of surgical complications. Patients with locally advanced rectal cancer receive three different treatments in our hospital, including long-course radiochemotherapy, short-course radiotherapy, and surgery directly. We can compare differences in their postoperative complications.

Research motivation

Some surgeons suspect that preoperative radiochemotherapy increases surgical complications, such as anastomotic leakage. As a result, surgeons are more likely to do additional diverting ileostomy for these patients. Our motivation is to determine if radiochemotherapy increases the incidence of complications or only increases the severity of complications. These findings can guide our treatment strategies.

Research objectives

To investigate surgical complications caused by three different preoperative radiotherapy regimens. It includes the incidence and severity of complications.

Research methods

This is a retrospective study. We analyzed 1197 patients with locally advanced rectal cancer between 2008 and 2010. Three hundred and forty-six patients were treated with preoperative long-course radiochemotherapy, and 259 patients received short-course radiotherapy (10 × 3 Gy) before surgery. The remaining 592 patients underwent total mesorectal excision (TME) alone without neoadjuvant therapy. The incidence of surgical complications was evaluated for up to 30 d after discharge from hospital. Severity was also studied according to Clavien–Dindo classification.

Research results

The major complications were anastomotic leakage and perineal wound complications. Both long-course and short-course radiotherapy were associated with incidence of anastomotic leakage, but the severity of anastomotic leakage did not increase in patients following preoperative radiotherapy. Temporary ileostomy can reduce incidence of anastomotic leakage. Compared with TME alone, short-course radiotherapy was associated with an increase in incidence and severity of perineal wound complications. Long-course radiotherapy seemed safe regarding this complication.

Research conclusions

Radiotherapy increased incidence but not severity of anastomotic leakage. Short-course radiotherapy was also accompanied with perineal wound complications. However, intervention appeared unnecessary to ameliorate the complications. The increase of complications seems to be acceptable. Our surgeons are more likely to use diverting ileostomy for patients with preoperative radiotherapy.

Research perspectives

We determined the advantages and disadvantages of preoperative radiotherapy, and this knowledge will inform our selection of different preoperative treatments. Our study is a retrospective study with a large sample size. In our opinion, a prospective randomized controlled study needs to be designed and performed.

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ORIGINAL ARTICLE

Retrospective Study

Histopathological characteristics of needle core biopsy and surgical specimens from patients with solitary hepatocellular carcinoma or intrahepatic cholangiocarcinoma

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Abstract

BACKGROUND

Pathological manifestations of hepatic tumours are often associated with prognosis. Although surgical specimens (SS) can provide more information, currently, pre-treatment needle core biopsy (NCB) is increasingly showing important value in understanding the nature of liver tumors and even in diagnosis and treatment decisions. However, the concordance of the clinicopathological characteristics and immunohistochemical (IHC) staining between NCB and SS from patients with hepatic tumours were less concerned.

AIM

To introduce a more accurate method for interpreting the IHC staining results in order to improve the diagnostic value of hepatic malignancy in NCB samples.

METHOD

A total of 208 patients who underwent both preoperative NCB and surgical resection for hepatocellular carcinoma (HCC) or intrahepatic cholangiocarcinoma

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RESULTS

Morphologically, the presence of compact tumour nests or a cord-like structure in NCB was considered the primary cause of misdiagnosis of HCC from ICC. The kappa statistic showed a moderate agreement in histomorphology (k = 0.504) and histological grade (k = 0.488) between NCB and SS of the tumours. A 4-tier (+++, ++, +, and -) scoring scheme that emphasized the focal neoplastic cell immunoreactivity of tumour cells revealed perfect concordance of CK19, GPC3 and HepPar1 between NCB and SS (k = 0.717; k = 0.768; k = 0.633). Furthermore, with the aid of a binary classification derived from the 4-tier score, a high concordance was achieved in interpreting the IHC staining of the three markers between NCB and final SS (k = 0.931; k = 0.907; k = 0.803), increasing the accuracy of NCB diagnosis C (k = 0.987; area under the curve = 0.997, 95%CI: 0.990-1.000; $P < 0.001$).

CONCLUSION

These findings imply that reasonable interpretation of IHC results in NCB is vital for improving the accuracy of tumour diagnosis. The simplified binary classification provides an easy and applicable approach.

Key words: Histopathological; Needle core biopsy; Surgical specimens; Solitary hepatocellular carcinoma; Intrahepatic cholangiocarcinoma

Core tip: Pathological manifestations of hepatic tumours are often associated with prognosis. The present study was designed to evaluate the concordance of the clinicopathological characteristics and staining of three biomarker between the needle core biopsy (NCB) and surgery specimen from patients with hepatocellular carcinoma or intrahepatic cholangiocarcinoma. Our results indicated that reasonable interpretation of immunohistochemical staining results in NCB is vital for improving the accuracy of tumour diagnosis. The simplified binary classification provides an easy and applicable approach to improve the diagnostic value of hepatic malignancy in NCB samples.

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INTRODUCTION

Liver malignancies including hepatocellular carcinoma (HCC) arising from hepatocytes and cholangiocarcinoma (CCA) arising from epithelial cells of the bile ducts are the fifth most commonly diagnosed tumours and the second most frequent cause of cancer-related deaths in males worldwide[1,2]. The mostly accepted view is that HCC and CCA are different diseases and have relatively independent characteristics in diseased populations and underlying diseases. In recent years, and perhaps until now, we have had somewhat naïve hopes of fitting the complex disease into a tidy and easily understood model. Currently, we classify malignancies mainly according to the anatomical site of the tumour, but the diagnosis of CCA is clinically difficult to identify with HCC, especially in tumour tissues obtained by needle core biopsy (NCB), which involves the combination of a variety of methods including imaging and biopsy pathology as well as Immunohistochemistry (IHC) techniques[3,4] based both on surgical specimens (SS) and NCB. To date, unfortunately, most CCA patients are usually diagnosed at a late stage of the disease. The overall 5-year survival of CCA is poor, and there is no sufficiently sensitive and specific biomarker available to facilitate the diagnosis or predict the effect of therapy.

With the in-depth understanding of the nature of this disease, simple anatomical division has not been able to explain the clinical manifestations of liver malignancies, and perhaps the whole course of the clinical manifestation has been determined at the

"origin" of the disease. International efforts of clinicians and scientists are helping to identify the genetic drivers of liver malignancy progression, which will unveil early diagnostic markers and direct the development of individualized therapies. Researchers must also face unprecedented challenges to distinguish the true genetic driver changes that are critical for tumour development to identify the most promising therapeutic target for liver malignancies.

Some novel diagnostic biomarkers, including cytokeratin-19 (CK19), glypican-3 (GPC3), and hepatocyte paraffin-1 (HepPar1), are considering to be related to the prognosis of patients with PCL as predictive markers based on pathological sections[5-8], which indicated that combining NCB, IHC, and imaging technology might help provide suggestions concerning patients' medical treatment selection and optimize therapeutic strategies at the time of diagnosis[9-12]. The purpose of this study was to compare the pathological characteristics of SS and NCB and attempt to improve the diagnostic rate of puncture specimens by immunohistochemical (IHC) scoring. It is hoped that our scoring system can be used in the future to facilitate early and accurate diagnoses in more patients.

MATERIALS AND METHODS

Subjects

The current study was conducted in accordance with the Declaration of Helsinki. All patients provided written, informed consent. The study was approved by the Local Ethics Committee. Between January 2008 and October 2015, 350 patients who underwent both preoperative NCB and surgical resection at Beijing You-An Hospital, Capital Medical University (163/350), Xijing Hospital, Fourth Military Medical University (116/350) and The Second Hospital, Hebei Medical University (71/350) were enrolled. All authors had evaluated original data and approved the final manuscript.208 patients were enrolled in the cohort for the present study (Figure 1).

NCB and surgical procedures and sample management

NCBs were performed under local anaesthesia with 2% lidocaine[13]. An automated biopsy gun (18-gauge core biopsy needle) was used to procure a minimum 1.5-cm-core biopsy specimen. All NCBs were performed using a similar similar fashionstand-alone protocol with computed tomography (CT) scans or ultrasound guidance to document the needle position within the lesion. In patients with a solitary nodule diameter greater than 5 cm, three different biopsies were performed within the lesion. When the tumour diameter was between 2-5 cm, two passes were usually performed, whereas when the tumour diameter was 2 cm or less, one pass was performed. The biopsy specimens were fixed in 10% neutral buffered formalin. All surgeries were performed by three independent groups of doctors. Criteria to evaluate the indication for surgical resection based on the liver function and tumour status have been described previously[14]. The formalin-fixed tissues ere processed, sectioned at a 5-μm thickness and stained with haematoxylin and eosin (H and E) technique.

IHC staining

Monoclonal antibody (clone BA17; 1:100) and mouse anti-human GPC3 monoclonal antibody (clone 1G12; 1:200) were purchased from the Zeta Company. Mouse anti-human monoclonal antibody HepPar1 (clone OCH1E5; 1:200) was purchased from Zymed Laboratories, Inc. The sections were steamed for 20 min in citrate target retrieval buffer (pH 6.0). Evidence for cytoplasmic staining of adjacent interlobular duct epithelia served as an internal positive control for CK19, yolk sac tumour tissue was used as a positive control sample for GPC3, and normal hepatocytes were used as positive control for HepPar1. Negative controls were established by substitution of the primary antibodies with non-immunized serum, resulting in the absence of signal detection.

Interpretation and diagnosis of the IHC results

Semi-quantitative scoring methods are widely used to convert the subjective perception of IHC marker expression by histopathologists into quantitative data, which are then used for statistical analyses and the establishment of conclusions. In the current study, two major approaches were used in the interpretation of IHC. The first approach was described by Sabattini *et al*[15]: "-" was denoted for a score of less than 10% positively stained tumour cells or no visible staining; "+" score was denoted for 10%–49% positive tumour cells; "++" was established for more than 50% positively stained tumour cells. The second approach was a four-tier semi-quantitative score method as described previously[16]: "-" for no labelling; "+" for positive cells scattered individually across the microscopic field; "++" for at least one

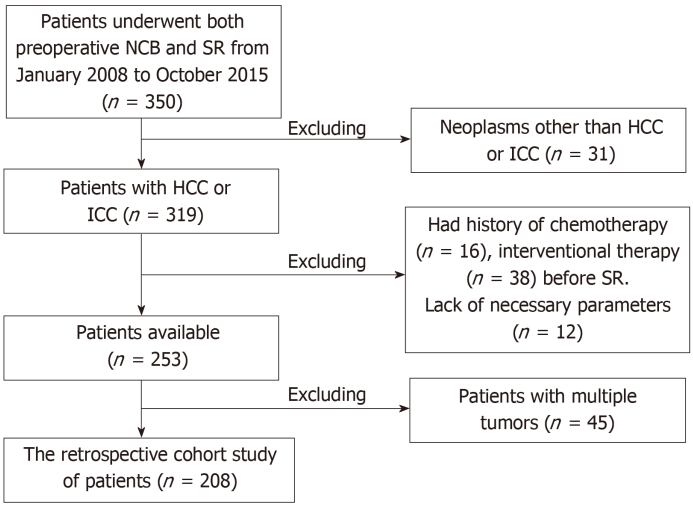

Figure 1 Schematic of the patient selection procedures. NCB: Needle core biopsy; SR: Surgical resection; HCC: Hepatocellular carcinoma; ICC: Intrahepatic cholangiocarcinoma.

positive cluster; and "+++" for numerous positive clusters present within the tumour (Figure 2). Considering the advantages of two-tier grading in diagnostic decisions, combining all positive reports of the three- or four-tier grading system into one group was further proposed.

In case A, HepPar-1 showed positive expression in one cluster (++) and GPC3 showed positive staining of approximately the entire tumour nest (+++) in NCB. However, both markers showed massive positive staining (+++) across all microscopic fields in SS. In case B, CK19 showed scattered positive cells or clusters (+++) in NCB. Although GPC3 showed scattered positive staining (+) in NCB, scattered positive individuals or positive clusters (+++) in SS could be observed. In case C, GPC3 expression showed two positive clusters around the sinusoid-like structures (++) in NCB, whereas massive positive clusters were observed across the microscopic field (+++) in SS.

The biopsy and SS were assessed independently and blindly by two investigators according to WHO criteria[17]. When two pathologists reached two different conclusions, a consensus was essential for the final results. Surgically resected specimens were used as the gold standard for diagnosis or histological grading. The ambiguous morphological appearances in NCB were diagnosed according to the above-described different scoring methods used for CK19, GPC3[18], and HepPar1 expression. To investigate the accuracy of diagnosis in NCB, diagnoses based on a single morphological evaluation or combining IHC and morphological observations in NCB were compared to the gold standard.

Histological grading

The degree of pathological differentiation of HCC was scored using the modified nuclear grading scheme outlined by Edmondson and Steiner[19]: G1–G2: well differentiated; G3: moderately differentiated; G4: poorly differentiated. In all cases, the tumour grade was defined by the poorest degree of differentiation identified within the tumour upon pathological analysis of the entire specimen.

Statistical analysis

Analyses were performed by IBM SPSS (version 22.0, SPSS, Chicago, IL). The data were expressed as the mean ± SD. χ^2 test and Student's *t* test were used to compare the distribution of categorical and continuous variables, respectively. To evaluate the ability of preoperative NCB to predict the final surgical pathological diagnosis of the tumours, a receiver operating characteristic curve analysis was used, and the area under the curve (AUC) was calculated to assess the performance of preoperative NCB. The similarities in expression of the biomarkers between NCB and SS were assessed using the kappa statistic: Kappa values < 0 indicated "no agreement", 0–0.20 "slight", 0.21–0.40 "fair", 0.41–0.60 "moderate", 0.61–0.80 "substantial", and 0.81–1 "almost" perfect agreement[20]. $P < 0.05$ was considered statistically significant.

RESULTS

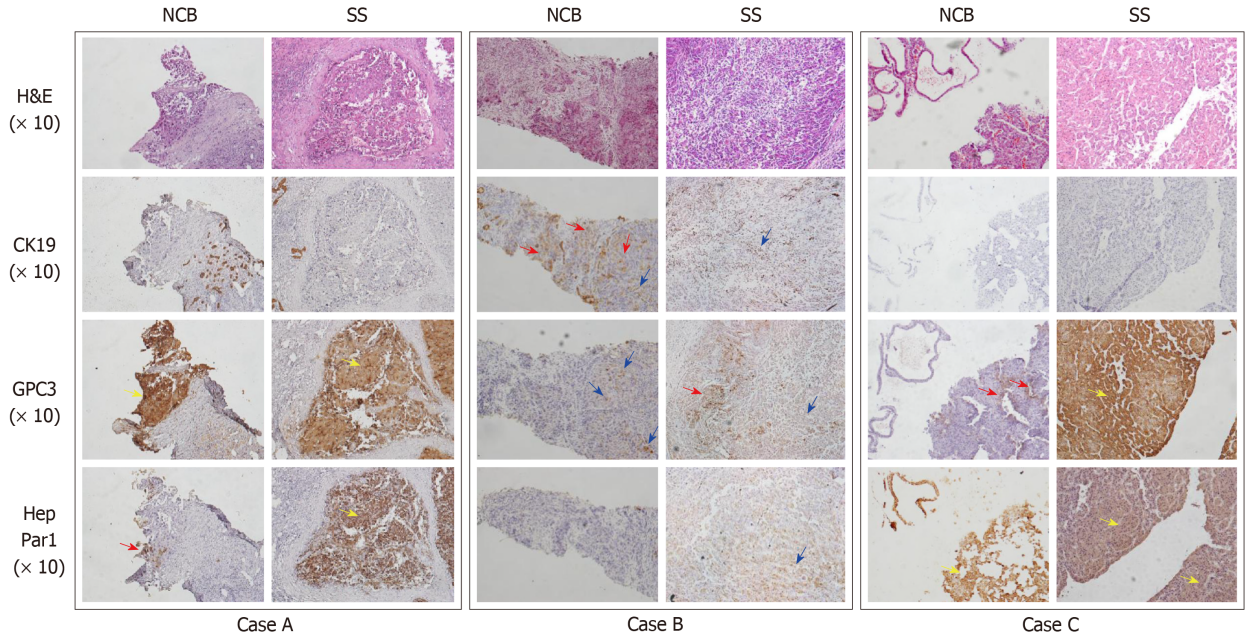

Figure 2 The four-tier score method for interpretation of the IHC staining of the three markers. NCB: Needle core biopsy; SS: Surgical specimen; H&E: Haematoxylin and eosin; CK: Cytokeratin; GPC3: Glypican 3; HepPar1: Hepatocyte paraffin-1; Blue arrow: Scattered positive cells; Red arrow: One positive cluster; Yellow arrow: Numerous positive clusters or massive positive staining.

Clinicopathological characteristics

The clinicopathological characteristics of the 208 patients are summarized in Table 1. The participants included 177 males and 31 females, with mean age of 54.37 ± 11.1 (26–84 and a mean tumour diameter of 4.6 ± 2.5 (1.8–15) cm. One hundred seventy-three patients (83.3%) exhibited cirrhosis, 192 (92.3%) had viral hepatitis B, and 12 (5.8%) presented hepatitis C infection, which accounted for the vast majority of the enrolled patients (98.1%). Complications of NCB were not recorded in this study.

Consistency of the morphology between SS and NCB

In this study, the kappa statistic showed that the agreement in histological subtypes between NCB and SS was 0.504 ($P < 0.001$) (Table 2). The result indicated that the degree of histological concordance between NCB and SS was moderate. Although histological disagreements between NCB and SS were also observed, most of the histological subtypes of PCL did not impact the accuracy of the NCB diagnosis, except the compact tumour nests/disordered cell mass and the cord-like structure in a fibrous stroma. Based solely on histomorphology, 7 (9.6%) intrahepatic cholangiocarcinoma (ICC) cases were misdiagnosed with HCC and 10 (6.4%) HCC cases were misdiagnosed with ICC among the NCB samples.

Consistency of CK19, GPC3, and HepPar1 detection between SS and NCB

GPC3 and HepPar1 are routine diagnostic markers for HCC.CK19 is also expressed in some subtypes of HCC[21,22] and hepatoblastoma[7], indicating that these tumours might originate from hepatic progenitor cells or hepatoblasts.

The four- and three-tier score method demonstrated a kappa value for consistency between NCB and SS of 0.717 (substantial) and 0.841 (almost perfect) for CK19, 0.768 (substantial) and 0.714 (substantial) for GPC3, and 0.633 (substantial) and 0.619 (substantial) for HepPar1, respectively. When the simplified two-tier score method (positive and negative) was used, the corresponding kappa value for CK19 was 0.931 and 0.979, for GPC3 was 0.907 and 0.933, and for HepPar1 was 0.803 and 0.874, in the 4-tier and 3-tier origin groups, respectively (Tables 3-5). The results indicated that both the 4-tier and 3-tier-based binary classifications had nearly perfect consistency in the interpretation of the IHC results between NCB and SS. Therefore, the two binary classification methods were used in further analyses.

Diagnostic accuracy of NCB

Based solely on morphology, the sensitivity, specificity, and accuracy of NCB in PCL diagnosis were 86.5%, 93.6%, and 91.8%, respectively. The AUC value of NCB was 0.901 (95%CI: 0.842-0.959; $P < 0.001$). The kappa statistic showed that the agreement between the NCB morphological diagnosis and gold standard was 0.786 ($P < 0.001$).

Table 1 Baseline clinical characteristics of the patients included in the study

Variable		Value
Age (yr)	Median	55
	Range	26-84
	means ± SD	54.4 ± 11.1
Sex, *n* (%)		
	Male	177 (85.1)
	Female	31 (14.9)
Cirrhosis, *n* (%)		
	Yes	173 (83.2)
	No	35 (16.8)
Aetiology, *n* (%)		
	HBV infection	192 (92.3)
	HCV infection	12 (5.8)
	alcohol abuse	2 (1.0)
	Primary biliary cirrhosis	1 (0.5)
	Schistosoma infection	1 (0.5)
Tumour diameter (cm)		
	Median	4.0
	Range	1.8-15
	means ± SD	4.6 ± 2.5

SD: Standard deviation; HBV: Hepatitis B virus; HCV: Hepatitis C virus.

When the 3-tier based binary classification was used, 7 (3.4%) cases were misdiagnosed in NCB. The sensitivity, specificity, and accuracy of the diagnostic method in NCB were 96.2%, 96.8%, and 96.6%, respectively. The AUC value was 0.965 (95%CI: 0.931-0.999; $P < 0.001$). The kappa statistic showed that the agreement between the NCB and SS diagnosis was 0.912 ($P < 0.001$). However, when the 4-tier based binary classification was used, only 1 (0.5%) case was misdiagnosed in NCB. The sensitivity, specificity, and accuracy of the method in NCB were 99.4%, 100%, and 99.5%, respectively. The AUC value was 0.997 (95%CI: 0.990-1.000; $P < 0.001$). The kappa statistic showed that the agreement between NCB and SS diagnosis was 0.987 ($P < 0.001$) (Figure 3 and Table 6).

Consistency of the histological grading between SS and NCB

The degree of concordance between NCB and the final SS tumour three-tier grade is shown in Table 7. In NCB, the HCC cases classified as well, moderately, and poorly differentiated represented 37 (17.8%), 99 (47.6%), and 72 (34.6%) of all cases, respectively. In contrast, in SS, there was a significantly lower proportion of well-differentiated HCC and a higher proportion of poorly differentiated HCC (well: 18/208, 8.7%; moderately: 93/208, 44.7%; poorly: 97/208, 46.6%) ($P < 0.05$). The corresponding kappa statistic for concordance was 0.488 ($P < 0.001$). Considering the significance of poorly differentiated HCC in prognostic predictions in comparison to individuals with well and moderately differentiated HCC, we further combined the well and moderately differentiated HCC into one group. The degree of consistency of the two-tier grade (well + moderately *vs* poorly) between SS and NCB was then increased but nevertheless remained at the moderate level (k = 0.558).

DISCUSSION

The accurate diagnosis of PCL is very important prior to surgical procedures, as well as for prognostication and information regarding future treatment decisions. Despite significant advances in non-invasive techniques, such as radiological imaging and serum tumour biomarker detection, preoperative NCB should be one of the most important approaches in diagnosis of PLC, especially the differentiation of HCC and ICC. In the present study, the concordance of the clinicopathological characteristics and CK19, GPC3, and HepPar1 IHC staining between NCB and SS in patients with solitary HCC or ICC was investigated. Our results showed different degrees of

Table 2 Degree of concordance of the morphology between needle core biopsy and surgery specimen

SS	NCB							
	A	B	C	D	E	F	G	H
A	21	0	0	0	0	0	0	0
B	16	51	1	0	1	0	0	0
C	2	14	36	0	0	0	0	0
D	2	3	1	2	0	0	0	0
E	0	5	4	0	3	0	0	0
F	0	0	0	0	0	2	0	0
G	0	0	21	1	0	0	11	9
H	0	0	0	0	0	0	2	2
Kappa value	0.504							
P value	< 0.001							

SS: Surgery specimen; NCB: Needle core biopsy; A: Acinar/pseudoglandular/thin trabecular; B: Thick trabecular; C: Compact tumour nests; D: Clear cell hepatocellular carcinoma; E: Scirrhous hepatocellular carcinoma; F: Spindle cell hepatocellular carcinoma; G: Glandular; H: Cord-like pattern

discrepancy in the histomorphology and CK19, GPC3, and HepPar1 detection of tumours between NCB and SS, which could impact the diagnostic accuracy and predispose patients towards an underestimated tumour grade and malignancy potential.

Morphological characteristics have been the source for the pathological diagnosis. Microscopically, the typical pathological patterns of HCC include trabecular, acinar, and pseudo-glandular features, whereas ICC shows adenocarcinomatous structures characterized by tubular complexes and a moderate amount of fibrous stroma[23]. However, HCC and ICC occasionally share overlapping morphological appearances, which can pose challenges in the differential diagnosis. In this study, the morphological observation led to a misdiagnosis rate of 8.2% in NCB, which was attributed to the presence of compact tumour nests or cord-like structures in tissues[24]. With the aid of IHC staining, the diagnostic accuracy in NCB significantly improved.

Although IHC can remarkably aid in pathological diagnosis, the heterogeneous expression of biomarkers in one tumour can directly interfere with the interpretation of IHC results and determine the variability of the achieved results, especially in tumour tissues obtained with needle biopsy[3,25-28]. Semi-quantitative scoring based on the proportion of positively stained tumour cells is widely used to convert IHC staining into positive or negative results, although varied cut points of 10%, 25% or 50% positive immunoreactivity in the specimen were used by different groups[29]. The usage of these methods led to a negligible significance of focal neoplastic cell immunoreactivity within a limited sample of carcinomas. In the current study, according to the 3-tier scoring, some cases with scattered positive tumour cells in CNB can be interpreted as negative, and therefore, a diametrically opposing positive or negative result can be obtained. In CNB diagnosis integrating CK19, GPC3 and HepPar1 IHC detection, semi-quantitative scoring based on the proportion of positively stained tumour cells should be avoided.

In comparison to 3-tier scoring, 4-tier scoring and the further proposed binary classification derived from 4-tier scoring showed a high concordance in interpreting the IHC staining of CK19, GPC3, and HepPar1 between NCB and SS, which also showed a decisive role in increasing the accuracy of diagnosis in CNB. Therefore, this simplified binary classification can be used as an easy and applicable approach in preoperative CNB diagnosis.

During the past decade, several studies have emphasized the significance of histological grading in the risk of recurrence or metastasis in HCC after liver resection or transplantation[30]. Therefore, the degree of concordance of NCB grade to the gold standard by SS will directly determine the safety and reliability of preoperative NCB grading in the evaluation of prognosis of patients and the treatment decision. Nevertheless, in the present study, the consistency of the three-tier histological grading between SS and NCB was moderate (k = 0.488). Since several previous studies have reported that poor differentiation of HCC was the independent prognostic indicator, we further compared the consistency of the histological grade between SS and NCB in a two-tier grading method (well and moderately vs. poorly). Our result

Table 3 Degree of concordance in the interpretation of CK19 staining between needle core biopsy and surgery specimen

NCB		SS										
		4-tier				4-tier-based binary classification		3-tier			3-tier-based binary classification	
		+++	++	+	-	+	-	++	+	-	+	-
4-tier	+++	52	1	0	0							
	++	3	2	0	0							
	+	3	21	4	0							
	-	0	0	7	115							
4-tier-based binary classification	+					86	0					
	-					7	115					
3-tier	++							35	8	0		
	+							7	22	0		
	-							0	2	134		
3-tier-based binary Classification	+										72	0
	-										2	134
Kappa value		0.717				0.931		0.814			0.979	
P value		< 0.001				< 0.001		< 0.001			< 0.001	

NCB: Needle core biopsy; SS: Surgery specimen.

showed that the degree of consistency of the two-tier histological grading between SS and NCB was increased; however, it continued at the moderate level (k = 0.558), which was similar to the results of another previous study (k = 0.380)[31]. Notably, although the agreement between SS and NCB histological grading was not as perfect as expected, the presence of the poorly differentiated region in NCB can still be valuable in prognosis prediction because the histological grading of tumours has been determined according to the worst differentiation clusters or regions microscopically.

There were some limitations in this study. Even with the aid of the 4-tier based binary classification, 0.48% (1/208) patients were misdiagnosed as preoperative NCB in this cohort, which indicated that the utility of the three markers in NCB had diagnostic limitations and that the combined utility of other biomarkers would be necessary to further improve the diagnostic accuracy. In addition, this study also does not address potential adverse events following NCB. Third, the present study investigated the concordance of detective indexes in patients with a solitary tumour between NCB and SS. The degree of consistency of the indicators in patients with a multifocal tumour between NCB and SS necessitates further investigation, which will be the focus of our future studies.

In conclusion, the present study suggested that in preoperative NCB, the presence of compact tumour nests or a cord-like structure alone were the main causes of misdiagnosis of HCC from ICC. Combining the detection of CK19, GPC3, and HepPar1 can improve the accuracy of diagnosis. However, focal neoplastic cell immunoreactivity of these markers in NCB should not be neglected. The concordance of histological grade between NCB and the SS was moderate. These findings imply that reasonable interpretation of IHC staining results and evaluation of the histomorphology in NCB are vital for improving the accuracy of tumour diagnosis, as well as prognostic prediction.

Table 4 Degree of concordance in the interpretation of glypican-3 staining between needle core biopsy and surgery specimen

NCB		SS										
		4-tier				4-tier-based binary classification		3-tier			3-tier-based binary classification	
		+++	++	+	-	+	-	++	+	-	+	-
4-tier	+++	62	1	0	0							
	++	18	25	2	0							
	+	2	2	15	0							
	-	0	1	8	72							
4-tier-based binary classification	+					127	0					
	-					9	72					
3-tier	++							42	5	0		
	+							25	26	5		
	-							0	2	103		
3-tier-based binary classification	+										98	5
	-										2	103
Kappa value			0.768			0.907			0.714		0.933	
P value			< 0.001			< 0.001			< 0.001		< 0.001	

NCB: Needle core biopsy; SS: Surgery specime.

Table 5 Degree of concordance in the interpretation of Hepatocyte paraffin-1 staining between needle core biopsy and surgery specimen

NCB		SS										
		4-tier				4-tier-based binary classification		3-tier			3-tier-based binary classification	
		+++	++	+	-	+	-	++	+	-	+	-
4-tier	+++	79	0	0	0							
	++	15	1	0	0							
	+	5	12	12	0							
	-	1	5	13	65							
4-tier-based binary classification	+					124	0					
	-					19	65					
3-tier	++							35	26	0		
	+							12	32	7		
	-							0	6	90		
3-tier-based binary classification	+										105	7
	-										6	90
Kappa value			0.633			0.803			0.619		0.874	
P value			< 0.001			< 0.001			< 0.001		< 0.001	

NCB: Needle core biopsy; SS: Surgery specimen.

Table 6 Degree of concordance between needle core biopsy and surgery specimen diagnosis

NCB diagnosis		SS diagnosis (gold standard)	
		HCC	ICC
Morphology	HCC	146	7
	ICC	10	45
	Kappa value	0.786	
	P value	< 0.001	
3-tier-based binary classification	HCC	151	2
	ICC	5	50
	Kappa value	0.912	
	P value	< 0.001	
4-tier-based binary classification	HCC	155	0
	ICC	1	52
	Kappa value	0.987	
	P value	< 0.001	

NCB: Needle core biopsy; SS: Surgery specimen; HCC: Hepatocellular carcinoma; ICC: Intrahepatic cholangiocarcinoma.

Table 7 Degree of concordance of the histological grade between surgery specimen and needle core biopsy

SS	NCB				
	3-tier			2-tier	
	Well	Moderately	Poorly	Well and moderately	Poorly
3-tier					
Well	15	3	0		
Moderately	17	66	10		
Poorly	5	30	62		
2-tier					
Well + moderately				101	10
Poorly				35	62
Kappa value		0.488		0.588	
P value		< 0.001		< 0.001	

SS: Surgery specimen; NCB: Needle core biopsy.

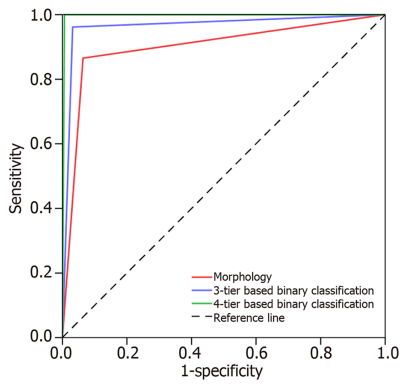

Figure 3 Receiver operating characteristic showing the accuracy of needle core biopsy diagnosis.

ARTICLE HIGHLIGHTS

Research background

Pathological manifestations of hepatic tumours are often associated with prognosis. Although surgical specimens (SS) can provide more information, currently, pre-treatment needle core biopsy (NCB) is increasingly showing important value in understanding the nature of liver tumors and even in diagnosis and treatment decisions. However, the concordance of the clinicopathological characteristics and immunohistochemical (IHC) staining between NCB and SS from patients with hepatic tumours were less concerned.

Research motivation

The present study was designed to evaluate the concordance of the clinicopathological characteristics and the novel biotic marker of CK19, GPC3, and HepPar1 staining between the NCB and SS from patients with hepatocellular carcinoma (HCC) or intrahepatic cholangiocarcinoma (ICC).

Research objectives

We want to introduce a more accurate method for interpreting the immunohistochemical staining results to improve the diagnostic value of hepatic malignancy in NCB samples.

Research methods

A total of 208 patients who underwent both preoperative NCB and surgical resection for HCC or ICC between 2008 and 2015 were enrolled in this study. The expression of CK19, GPC3, and HepPar1 were detected by IHC staining. Clinicopathological, NCB, and surgical data were collected and analysed using χ^2 and kappa statistics.

Research results

Morphologically, the presence of compact tumour nests or a cord-like structure in NCB was considered the primary cause of misdiagnosis of HCC from ICC. The kappa statistic showed a moderate agreement in histomorphology (k = 0.504) and histological grade (k = 0.488) between NCB and SS of the tumours. A 4-tier (+++, ++, +, and -) scoring scheme that emphasized the focal neoplastic cell immunoreactivity of tumour cells revealed perfect concordance of CK19, GPC3 and HepPar1 between NCB and SS (k = 0.717; k = 0.768; k = 0.633). Furthermore, with the aid of a binary classification derived from the 4-tier score, a high concordance was achieved in interpreting the IHC staining of the three markers between NCB and final SS (k = 0.931; k = 0.907; k = 0.803), increasing the accuracy of NCB diagnosis C(k = 0.987; area under the curve = 0.997, 95%CI: 0.990-1.000; $P < 0.001$).

Research conclusions

Our findings imply that reasonable interpretation of IHC staining results in NCB is vital for improving the accuracy of tumour diagnosis. The simplified binary classification provides an easy and applicable approach.

Research perspectives

Although the binary classification can significantly improve the accuracy of diagnosis of HCC or ICC, it is unclear whether the method can be transferred to patients with other tumors. In addition, the degree of consistency of the indicators in patients with a multifocal tumor between NCB and SS necessitates further investigation, which will be the focus of our future studies.

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ORIGINAL ARTICLE

Retrospective Study

Surgical resection of gastric stump cancer following proximal gastrectomy for adenocarcinoma of the esophagogastric junction

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Author contributions: Tian YT and Xue LY designed the research; Ma FH, Li WK and Chen YT analyzed the data and wrote the paper; Li Y, Kang WZ, Xie YB, Zhong YX and Xu Q collected the patient's clinical data.

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Informed consent statement: The need for informed consent was waived due to the retrospective nature of the study, and the data were anonymously analyzed.

Conflict-of-interest statement: The authors declare that they have no conflicts of interest.

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Abstract

BACKGROUND

Proximal gastrectomy (PG) is performed widely as a function-preserving operation for early gastric cancer located in the upper third of the stomach and is an important function-preserving approach for esophagogastric junction (EGJ) adenocarcinoma. The incidence of gastric stump cancer (GSC) after PG is increasing. However, little is known about the GSC following PG because very few studies have been conducted on the disease.

AIM

To clarify clinicopathologic features, perioperative complications, and long-term survival rates after the resection of GSC following PG.

METHODS

Data for patients with GSC following PG for adenocarcinoma of the EGJ diagnosed between January 1998 and December 2016 were retrospectively reviewed. Multivariate analysis was performed to identify factors associated with overall survival (OS). GSC was defined in accordance with the Japanese Gastric Cancer Association.

RESULTS

A total of 35 patients were identified. The median interval between the initial PG

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and resection of GSC was 4.9 (range 0.7-12) years. In 21 of the 35 patients, the tumor was located in a nonanastomotic site of the gastric stump. Total gastrectomy was performed in 27 patients; the other 8 underwent partial gastrectomy. Postoperative complications occurred in 6 patients (17.1%). The tumor stage according to the depth of tumor invasion was T1 in 6 patients, T2 in 3 patients, T3 in 9 patients, and T4 in 17 patients. Lymph node metastasis was observed in 18 patients. Calculated 1-, 3-, and 5-year OS rates were 86.5%, 62.3%, and 54.2%, respectively. Multivariate analysis showed advanced T stage to be associated with OS.

CONCLUSION
This study reveals the characteristics of GSC following PG for adenocarcinoma of the EGJ and suggests that a surgical approach can lead to a satisfactory outcome.

Key words: Gastric stump cancer; Proximal gastrectomy; Esophagogastric junction; Distal gastrectomy

Core tip: The clinicopathological characteristics, treatment, and prognosis of gastric stump cancer (GSC) after distal gastrectomy have been well investigated, however, there is limited information on GSC after proximal gastrectomy (PG). We revealed characteristics of GSC in detail using the largest number of patients to date. Our results suggest that surgical approaches can achieve satisfactory outcomes in GSC following PG. The factor associated with OS based on multivariate analysis was advanced T stage and GSC is more likely to be diagnosed at an advanced stage. Thus, endoscopic follow-up of the gastric stump should be conducted to detect GSC at an early stage.

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INTRODUCTION

Although the prevalence of gastric cancer is decreasing, the incidence of esophagogastric junction (EGJ) adenocarcinoma is increasing[1]. The choice of surgical techniques for EGJ adenocarcinoma is controversial, yet proximal gastrectomy (PG) remains an important surgical option[2,3]. PG is also widely used as a function-preserving approach for early-stage proximal stomach cancer[4]. The incidence of gastric stump cancer (GSC) after PG is growing[5-8], and GSC following PG may thus be increasingly encountered by surgeons in the coming years.

The clinicopathological characteristics, treatment, and prognosis of GSC after distal gastrectomy (DG) have been well investigated[9-11]; however, there is limited information on GSC after PG. To our knowledge, there are only few studies have been published on GSC following PG[8,12,13]. As such, we conducted a single-center retrospective study to understand the associated clinicopathological features, surgical results and long-term outcomes of GSC following PG.

MATERIALS AND METHODS

This retrospective study was approved by the Institutional Review Board of Cancer Hospital of the Chinese Academy of Medical Sciences and was based on demographic and pathological data retrieved from a computerized database of all patients with gastric cancer treated at this facility between January 1998 and December 2016. The need for informed consent was waived due to the retrospective nature of the study, and the data were anonymously analyzed.

PG or PG plus esophagectomy followed by esophagogastrostomy reconstruction

are usually indicated for EGJ adenocarcinoma and gastric cancer located in the upper third of the stomach. We defined GSC according to the Japanese Classification and Treatment Guidelines for Gastric Cancer (14th edition), in which GSC is defined as a cancer arising in the gastric stump after gastrectomy, regardless of the histology of the previous lesion (benign or malignant), risk of recurrence, extent of initial resection, or method of reconstruction[14]. Thirty-five patients who had undergone resection of the gastric stump for GSC following PG at Cancer Hospital of the Chinese Academy of Medical Sciences were eligible for inclusion in the study.

To investigate whether the time interval significantly influenced survival, we divided the patients into 2 groups: Interval < 5 years (n = 21) and ≥ 5 years (n = 14). We also divided the patients into 2 groups to investigate whether the tumor location significantly impacted survival: Tumors located in anastomotic sites (n = 14) and tumors located in nonanastomotic sites (n = 20). Medical records were reviewed with regard to preoperative medical conditions, perioperative complications, histopathological results and follow-up data.

Statistical analysis

Cumulative survival rates were obtained using the Kaplan–Meier method and compared using the log-rank test to evaluate statistically significant differences. Cox proportional hazards regression analysis was used to evaluate factors affecting overall survival (OS). $P < 0.05$ was considered significant. The statistical analysis was performed with SPSS for Windows version 22.0.

RESULTS

Patients and clinical characteristics

The demographic and clinical characteristics of 35 patients with GSC following PG are shown in Table 1. The mean age was 60 ± 11 years, and the male-to-female ratio was 7.75 to 1. Of the 35 patients, the mean interval between primary PG and the development of GSC was 4.9 (range 0.7-12) years. The time to treatment of GSC was within 5 years in 21 patients, within 5–10 years in 10 patients, and longer than 10 years in 4 patients. Regarding the initial EGJ adenocarcinoma, 2 patients had type I, 29 had type II, and 4 had type III disease according to Siewert Classification. All of the patients underwent PG with esophagogastrostomy. With regard to the site of tumors in the gastric stump, 14 and 21 were in anastomotic and nonanastomotic sites, respectively.

Surgical characteristics and short-term outcomes

Total gastrectomy as the primary procedure for GSC was performed in 27 patients (77.1%) of all patients; partial resection of the gastric remnant was performed in 8 patients (22.9%). In 5 patients, resection of one or more adjacent organs was performed together with gastrectomy. The mean operation time was 343 ± 132 min. The mean intraoperative blood loss volume was 513 ± 383 ml. Postoperative complications were detected in 6 patients (17.1%): 4 patients developed leakage from the anastomotic site, 1 developed wound infection, 1 developed hemorrhage, and 1 developed postoperative ileus. However, none of these patients died (Table 2).

Histopathological characteristics

Histological analysis revealed 26 adenocarcinomas and 9 adenocarcinomas with signet ring cells. Analysis of histological differentiation revealed 3 well-differentiated tumor types, 11 moderately differentiated tumor types, and 21 poorly differentiated tumor types. The disease stage according to the depth of tumor invasion was T1 in 6 patients, T2 in 3 patients, T3 in 9 patients, T4a in 11 patients, and T4b in 6 patients. The median number of dissected lymph nodes was 11.1 ± 7.4, and the median number of lymph node metastases was 2.9 ± 4.2. Lymph node metastasis was observed in 18 patients (Table 3).

Long-term outcomes and factors affecting survival

The 1-, 3-, and 5-year OS rates were 86.5%, 62.3%, and 54.2%, respectively. The results of the Cox proportional hazards model demonstrated T stage to be a significant independent prognostic factor for survival (Table 4). The 5-year survival rates for patients with T1/T2, T3 and T4 disease were 85.7%, 72.0% and 30.6%, respectively.

DISCUSSION

GSC was originally defined as gastric cancer occurring at least five years after after

Table 1 Clinical characteristics of patients

Characteristics		Number of patients (%)
Sex	Male	31 (88.6)
	Female	4 (11.4)
Age (yr)		60 ± 11 (33-83)
ASA	I-II	25 (71.4)
	III-IV	10 (28.6)
Comorbidity	Any comorbidity	7 (20)
	Hypertension	2 (5.7)
	Diabetes	1 (2.9)
	COPD	1 (2.9)
	Coronary artery disease	2 (5.7)
	Cerebral vascular disease	1 (2.9)
Family history of gastric cancer		4 (11.8)
Siewert type of initial EGJ adenocarcinoma	SiewertI	2 (5.7)
	Siewert II	29 (82.9)
	Siewert III	4 (11.4)
Adjuvant therapy after initial operation	Received	17 (48.6)
	Not received	4 (11.4)
	Unknown	14 (40)
Tumor location	Anastomotic site	14 (40)
	Nonanastomotic site	21 (60)
Interval (yr)		4.9 ± 3.2 (0.7-12)
Interval	< 5 yr	21 (60)
	≥ 5 yr, < 10 yr	10 (28.6)
	≥ 10 yr	4 (11.4)

COPD: Chronic obstructive pulmonary disease; EGJ: Esophagogastric junction.

DG for benign disease[15,16]. Recently, GSC has been used to refer to all cancers detected in the gastric stump, irrespective of the primary disease or initial operation[17]. The incidence of GSC following PG is increasing, and that of GSC is reportedly higher after PG (3.6%–9.1%) than after DG (0.4%–2.5%)[18]. Moreover, Nozaki *et al*[19] found that PG is an independent risk factor for GSC. Compared to DG, PG may result in an additional risk for GSC[11]. Surgery, pathogenesis, and prognosis of GSC after DG are well investigated; however, little is known about GSC following PG because very few studies have been conducted on the disease. To the best of our knowledge, this is the first study investigating GSC following PG for EGJ adenocarcinoma.

Resection of GSC is associated with intra-abdominal adhesion after the initial procedure. Surgeons sometimes encounter technical difficulties during resection, which leads to prolonged operation time and excessive blood loss. Furthermore, intraoperative surgical complications, such as intestinal injury, may occur. Previous studies have reported an overall surgical complication rate of 19%-47% for GSC, with operation-related mortality rates of 2%-13%[20]. However, little is known about the complication rate of GSC following PG. In our study, the overall complication rate was 17.1%, which is relatively low. Additionally, 5 of 35 patients (14.3%) required additional organ resection; this rate is also lower than that reported for GSC after DG[21]. The need for additional organ resection may complicate surgery in patients with GSC.

Ohyama *et al*[12] identified almost the same numbers of differentiated and undifferentiated tumors in GSC. However, in our study, 21 of 35 tumors were poorly differentiated. Because only a few studies have been published on the pathological type of GSC, the characteristics of this disease remain unclear. In the present study, early GSC was diagnosed in 6 (17%) of 35 patients, whereas T4 disease was identified in 17 (48.6%). As GSC is more likely to be diagnosed at an advanced stage, endoscopic follow-up of the gastric stump is necessary to detect GSC at an early stage. The incidence of metastasis to lymph nodes was 54.3% (19/35) in the present study, which is higher than that of GSC after DG[11].

Although the number of patients in our study was small, the results showed a 5-

Table 2 Surgical characteristics and short-term outcomes

Surgical characteristics		Number of patients (%)
Operation type	Total gastrectomy	27 (77.1)
	Partial gastrectomy	8 (22.9)
Additional organ resection	Yes	5 (14.3)
	Yes	5 (14.3)
Estimated blood loss (mL)		513 ± 383
Operation time (min)		343 ± 132
Blood transfusion	No	9 (25.7)
	Yes	26 (74.3)
Postoperative complications	Any complication	6 (17.1)
	Leakage	4 (11.4)
	Hemorrhage	1 (2.9)
	Ileus	1 (2.9)
	Wound infection	1 (2.9)
Postoperative hospital stay (d)		18.4 ± 12.1

year OS rate of 54.2%. In addition, the results of the Cox proportional hazards model showed only T stage to be a significantly independent prognostic factor for survival. In contrast, the time interval or location did not affect survival, which may partly justify the definition of GSC, whereby time interval, tumor location and method of reconstruction are not considered.

Nevertheless, this study has several limitations. First, because not all patients in our study underwent PG at Cancer Hospital of the Chinese Academy of Medical Sciences, some important information on the initial operation was missing, such as the extent of lymphadenectomy, histology type, and pathologic stage. Second, the number of patients enrolled was small, mostly because of the rarity of the disease. Third, there is no standardized definition of GSC, which make our study not able to be accurately compared with previous studies on GSC.

Here, we reveal characteristics of GSC following PG in detail with the largest number of patients to date.Our results suggest that surgical approaches can achieve satisfactory outcomes in GSC following PG, similar to those in patients with typical gastric cancer. GSC is more likely to be diagnosed at an advanced stage, and thus, endoscopic follow-up of the gastric stump should be conducted to detect GSC at an early stage. Further larger-scale studies are necessary to clarify the characteristics of the disease.

Table 3 Histopathological characteristics

Pathological characteristics		Number of patients (%)
Histology	Adenocarcinoma	26 (74.3)
	Adenocarcinoma with signet ring cell	9 (25.7)
Pathologic grade	Poor	21 (60.0)
	Moderate	11 (31.4)
	Well	3 (8.6)
T stage	T1a-1b	6(17.1)
	T2	3 (8.6)
	T3	9 (25.7)
	T4a	11 (31.5)
	T4b	6 (17.1)
Number of dissected lymph nodes		11.1 ± 7.4
Number of lymph node metastasis		2.9 ± 4.2
N stage	N0	16 (45.7)
	N1/N2/N3	19 (54.3)

Table 4 Univariate and Multivariate analyses of clinicopathologic factors associated with overall survival

	5-yr OS (%)	Univariate		Multivariate	
		Hazard ratio	*P* value	Hazard ratio	*P* value
Sex: Male *vs* female	49.4 *vs* 66.7	3.352 (0.420-26.755)	0.229	-	-
Age: < 65 yr *vs* ≥ 65 yr	63.5 *vs* 44.7	0.597 (0.199-1.796)	0.354	-	-
Tumor location: Anastomotic *vs* nonanastomotic	57.7 *vs* 56.2	0.868 (0.265-2.846)	0.816	-	-
Interval: < 5 yr *vs* ≥ 5 yr	56.8 *vs* 55.4	0.665 (0.213-2.074)	0.479	-	-
Operation type: Completion gastrectomy *vs* segmental resection	56.2 *vs* 68.6	2.112 (0.464-9.614)	0.323	-	-
Histology: Adenocarcinoma *vs* adenocarcinoma with signet ring cell	65.6 *vs* 0	0.368 (0.104-1.306)	0.108	0.376 (0.098-1.44)	0.154
Pathologic grade: Poor *vs* moderate/well	49.7 *vs* 65.3	1.232 (0.401-3.786)	0.715	-	-
T stage: T1-3 *vs* T4	77.0 *vs* 30.6	0.144 (0.039-0.534)	**0.001**	0.166 (0.041-0.672)	**0.012**
N stage: N0 *vs* N+	73.8 *vs* 39.2	0.216 (0.058-0.807)	**0.013**	0.432 (0.103-1.822)	0.253

OS: Overall survival.

ARTICLE HIGHLIGHTS

Research background

Proximal gastrectomy (PG) is performed widely as a function-preserving operation for early gastric cancer located in the upper third of the stomach and is an important function-preserving approach for esophagogastric junction (EGJ) adenocarcinoma. The incidence of gastric stump cancer (GSC) after PG is increasing. However, little is known about the GSC following PG because very few studies have been conducted on the disease. To our knowledge, there are only few studies have been published on GSC following PG.

Research motivation

The clinicopathological characteristics, treatment, and prognosis of GSC after distal gastrectomy have been well investigated; however, there is limited information on GSC after PG. As such, we conducted a single-center retrospective study to understand the associated clinicopathological features, surgical results and long-term outcomes of GSC following PG.

Research objectives

The aim of this study is to clarify clinicopathologic features, perioperative complications, and long-term survival rates after resection of GSC following PG. We revealed characteristics of GSC following PG in detail with the largest number of patients to date.

Research methods

This is a retrospective study. Thirty-five patients who had undergone resection of the gastric stump for GSC following PG at Cancer Hospital of the Chinese Academy of Medical Sciences were eligible for inclusion in the study. Medical records were reviewed with regard to

preoperative medical conditions, perioperative complications, histopathological results and follow-up data. Cumulative survival rates were obtained using the Kaplan–Meier method and compared using the log-rank test to evaluate statistically significant differences. Cox proportional hazards regression analysis was used to evaluate factors affecting overall survival (OS).

Research results

This study reveals the characteristics of GSC following PG for adenocarcinoma of the EGJ and suggests that a surgical approach can lead to a satisfactory outcome. GSC is more likely to be diagnosed at an advanced stage, and thus, endoscopic follow-up of the gastric stump should be conducted to detect GSC at an early stage. Further larger-scale studies are necessary to clarify the characteristics of the disease.

Research conclusions

We revealed the characteristics of GSC following PG for adenocarcinoma of the EGJ and suggests that a surgical approach can lead to a satisfactory outcome. GSC is more likely to be diagnosed at an advanced stage, and thus, endoscopic follow-up of the gastric stump should be conducted to detect GSC at an early stage. The incidence of GSC after PG is increasing. Surgical approach can lead to a satisfactory outcome. This is the first study investigating GSC following PG for EGJ adenocarcinoma. GSC following PG should be compared with initial distal gastric cancer. We defined GSC according to the Japanese Classification and Treatment Guidelines for Gastric Cancer (14th edition). Our results suggest that surgical approaches can achieve satisfactory outcomes in GSC following PG, similar to those in patients with typical gastric cancer. There are only few studies have been published on GSC following PG. This study reveals the characteristics of GSC following PG for adenocarcinoma of the EGJ. Endoscopic follow-up of the gastric stump should be conducted to detect GSC at an early stage. Surgical approach should be performed for patients with GSC following PG.

Research perspectives

The factor associated with OS based on multivariate analysis was advanced T stage and GSC is more likely to be diagnosed at an advanced stage. Thus, endoscopic follow-up of the gastric stump should be conducted to detect GSC at an early stage.

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ORIGINAL ARTICLE

Observational Study

ADAMTS13 and von Willebrand factor are useful biomarkers for sorafenib treatment efficiency in patients with hepatocellular carcinoma

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Abstract

BACKGROUND

Many advanced hepatocellular carcinoma (HCC) patients are receiving sorafenib treatment. Sorafenib reportedly improves overall survival (OS) significantly in patients with HCC. Prediction of sorafenib response and prognosis in patients with HCC receiving sorafenib treatment are important due to the potentially serious side effects of sorafenib. A disintegrin-like and metalloproteinase with thrombospondin type-1 motifs 13 (ADAMTS13) and von Willebrand factor (VWF) are associated with the pathophysiology of liver cirrhosis and HCC through their roles in hypercoagulability; they are also associated with angiogenesis *via* vascular endothelial growth factor (VEGF). The imbalance between ADAMTS13 and VWF was associated with prognosis of various cancers in patients undergoing chemotherapy.

AIM

To investigate ADAMTS13 and VWF as potential biomarkers for sorafenib response and prognosis in patients with HCC receiving sorafenib treatment.

METHODS

Forty-one patients with HCC receiving sorafenib treatment were included in this study. The initial daily sorafenib dose was 400 mg in all patients. ADAMTS13 activity (ADAMTS13:AC), VWF antigen (VWF:Ag), VEGF levels were determined by enzyme-linked immunosorbent assay. Univariate and

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multivariate analyses were used to determine predictive factors for sorafenib response and prognosis in patients with HCC receiving sorafenib treatment.

RESULTS

ADAMTS13:AC was significantly higher in patients with stable disease (SD), partial response (PR), and complete response (CR) than in those with progressive disease (PD) ($P < 0.05$). In contrast, VWF:Ag and the VWF:Ag/ADAMTS13:AC ratio were significantly lower in patients with SD, PR, and CR than in those with PD ($P < 0.05$ for both). Multivariate analysis showed that the VWF:Ag/ADAMTS13:AC ratio was the only predictive factor for sorafenib response and ADAMTS13:AC was the only prognostic factor in patients with HCC receiving sorafenib treatment. The patients with a low ADAMTS13:AC (< 78.0) had significantly higher VEGF levels than those with a high ADAMTS13:AC (≥ 78.0) ($P < 0.05$).

CONCLUSION

The VWF:Ag/ADAMTS13:AC ratio and ADAMTS13:AC are potentially useful biomarkers for sorafenib response and prognosis, respectively, in patients with HCC receiving sorafenib treatment.

Key words: ADAMTS13; Von Willebrand factor; Biomarkers; Hepatocellular carcinoma; Sorafenib

Core tip: There is an urgent clinical need to prediction of sorafenib response and prognosis in patients with hepatocellular carcinoma (HCC) receiving sorafenib treatment due to the potentially serious side effects of sorafenib in these patients. Multivariate analysis showed that the von Willebrand factor (VWF) antigen (VWF:Ag)/a disintegrin-like and metalloproteinase with thrombospondin type-1 motifs 13 (ADAMTS13) activity (ADAMTS13:AC) ratio was the only predictive factor for sorafenib response and ADAMTS13:AC was the only prognostic factor in patients with HCC receiving sorafenib treatment. The VWF:Ag/ADAMTS13:AC ratio and ADAMTS13:AC are potentially useful biomarkers for sorafenib response and prognosis, respectively, in patients with HCC receiving sorafenib treatment.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most common cancer and the second most common cause of cancer-related deaths worldwide[1,2]. In Japan, medical treatment policies for HCC are based on the consensus-based clinical practice guidelines for HCC management set by the Japan Society of Hepatology (JSH)[3], which recommend that patients with HCC who cannot undergo liver resection, radiofrequency ablation, or transcatheter arterial chemoembolization (TACE) should be considered to receive molecularly targeted drugs including sorafenib[3]. Sorafenib is a small inhibitor of several tyrosine protein kinases, including vascular endothelial growth factor (VEGF) receptor, platelet derived growth factor (PDGF) receptor, and Raf family kinases[4]. Sorafenib was shown to significantly improve overall survival (OS) in patients with HCC[4]. It is important to predict response to sorafenib and prognosis of HCC patients treated with sorafenib to avoid ineffective treatments because sorafenib has various side effects including hand-foot syndrome.

A disintegrin-like and metalloproteinase with thrombospondin type-1 motifs 13 (ADAMTS13) is a metalloproteinase that specifically cleaves multimeric von Willebrand factor (VWF) between Tyr1605 and Met1606 residues in the A2 domain[5-8].

ADAMTS13 is produced exclusively in hepatic stellate cells adjacent to endothelial cells[9]. VWF is synthesized in vascular endothelial cells and released into plasma as unusually large VWF multimers[10]. In the presence of an imbalance between ADAMTS13 and VWF, VWF multimers are cleaved improperly, leading to their accumulation and induction of platelet thrombus formation under high-shear stress in microvessels[11].

We previously reported that the imbalance of ADAMTS13 and VWF was associated with the pathophysiology of liver cirrhosis (LC) and HCC[8,12,13], suggesting that LC and HCC might be related to hypercoagulability[8,12,13]. Previous studies have reported that the imbalance of ADAMTS13 and VWF is associated with angiogenesis through VEGF[14-17], which in turn is associated with LC and HCC development[18-20]. In addition, the imbalance of ADAMTS13 and VWF might be associated with sorafenib treatment efficiency because VEGF is inhibited by sorafenib[4]. Furthermore, blood coagulation cascade was demonstrated to be related to cancer development[21,22], indicating that the imbalance of ADAMTS13 and VWF is associated with hypercoagulability as well as cancer development[23]. Recent studies have also reported that the imbalance between ADAMTS13 and VWF was associated with prognosis of various cancers in patients undergoing chemotherapy[24].

In the current study, we investigated the relationship between plasma ADAMTS13 and VWF levels in patients with HCC receiving sorafenib treatment and determined whether plasma ADAMTS13 and VWF levels were useful biomarkers for prediction of sorafenib response and prognosis of HCC in patients with HCC receiving sorafenib treatment.

MATERIALS AND METHODS

Patients

There were 44 patients with HCC who were initiated on sorafenib treatment from December 2012 to November 2017 at our hospital. After excluding three patients who discontinued sorafenib treatment in the first month, 41 patients were included in this study. The initial daily sorafenib dose was 400 mg in all patients, and sorafenib therapy are based on the JSH consensus-based clinical practice guidelines for the management of HCC[3]. All patients underwent dynamic computed tomographic scanning or dynamic magnetic resonance imaging before sorafenib treatment, at 1 mo after starting sorafenib treatment, and every 3 mo thereafter. Radiologic response to therapy was evaluated according to modified response evaluation criteria in solid tumors[25]. Tumor-node-metastasis (TNM) stage was evaluated according to the TNM classification of the Union for the International Cancer Control of malignant tumors. No patient had infection, uncontrolled ascites, uncontrolled hepatic encephalopathy, or uncontrolled gastroesophageal varices. This study was approved by the local ethics committee of Nara Medical University and performed in accordance with the ethical standards stated in the Declaration of Helsinki. Informed consent was obtained from all participants included in the study.

Determination of ADAMTS13 activity and VWF antigen levels

Blood samples were collected from all patients at the time of admission, during their hospital stay or during regular outpatient treatment within 1 mo before sorafenib treatment initiation. The samples were stored in plastic tubes containing 0.38% v/v sodium citrate. Platelet-poor plasma, which was prepared by centrifuging the samples at 3000 *g* at 4 °C for 15 min, was stored as aliquots at −80 °C until analysis. Plasma ADAMTS13 activity (ADAMTS13:AC) was determined by a sensitive chromogenic enzyme-linked immunosorbent assay (Kainos Laboratories, Tokyo, Japan)[26]. Mean normal ADAMTS13:AC level was 99% ± 22%. Plasma VWF antigen (VWF:Ag) was measured by sandwich enzyme-linked immunosorbent assay using a rabbit anti-human VWF polyclonal antiserum (Dako, Glostrup, Denmark). Mean normal VWF:Ag level was 102% ± 33%[27].

Measurement of VEGF and VEGFR-2 levels

VEGF and VEGF receptor 2 (VEGFR-2) levels were determined by commercially available immunoassay kits (RayBiotech, United States, and R and D Systems, United States, respectively). The detection limits for VEGF and VEGFR-2 were 10 and 11.4 pg/mL, respectively.

Statistical analysis

Differences between groups were analyzed using the Mann–Whitney *U*-test, and correlations were calculated with Spearman's rank test. Categorical data were analyzed using Fisher's exact test. Univariate and multivariate analyses were per-

formed for predictive and prognostic factors for sorafenib treatment in HCC. Logistic regression analysis with stepwise selection of variables was performed to determine independent predictive factors of sorafenib treatment for HCC, and the Cox proportional hazards regression analysis with stepwise selection of variables was conducted to determine independent prognostic factors of sorafenib treatment for HCC. Progression-free survival (PFS) and OS curves were calculated using the Kaplan–Meier method, and differences between groups were assessed using the log-rank test. Data were expressed as medians with interquartile ranges. A two-tailed *P* value of less than 0.05 was considered statistically significant. Analyses were performed using EZR (Saitama Medical Center, Jichi Medical University), which is a graphical user interface for R (The R Foundation for Statistical Computing, version 2.13.0). Specifically, EZR is a modified version of R commander (version 1.6-3) that includes statistical functions that are frequently used in biostatistics[28].

RESULTS

Clinical characteristics of the patients

The patient characteristics are shown in Table 1. The median age of patients with HCC was 74.0 (69.0–81.0) years. The study population comprised 38 males and three females. Among these, 7, 20, 3, and 11 patients had hepatitis B virus, hepatitis C virus, non-alcoholic steatohepatitis, and alcohol abuse, respectively. The median maximum tumor size was 3.3 (2.5–7.7) cm. In this cohort, 3, 2, 1, and 33 patients had 1, 2, 3, and ≥ 4 tumors, respectively, whereas two patients had only distant metastases. Portal vein tumor thrombosis and distant metastasis were present in 7 and 17 patients, respectively. Serum levels of alpha-fetoprotein (AFP), des-γ-carboxy prothrombin (DCP), *lens culinaris* agglutinin-reactive fraction of AFP (AFP-L3%), VEGF, and VEGFR-2 were 121.8 (11.3–2611.0) ng/mL, 359.5 (58.0–5277.5) mAU/mL, 13.2 (1.7–42.4)%, 25.8 (14.1–40.1) pg/mL, and 6500 (5750–7400) pg/mL, respectively. DCP was directly correlated with VEGF ($r = 0.503$, $P < 0.05$). However, DCP was not correlated with VEGFR-2, and AFP or AFP-L3% was not correlated with VEGF or VEGFR-2. In the current study cohort, there were no differences in the characteristics of patients with stable disease (SD), partial response (PR), and complete response (CR) compared with those with progressive disease (PD), except the DCP levels and observation (survival) period.

Plasma ADAMTS13:AC and VWF:Ag level in patients with HCC receiving sorafenib treatment

ADAMTS13:AC level was significantly higher in patients with HCC who had SD, PR, and CR than those with PD ($P < 0.05$) (Figure 1A). In contrast, VWF:Ag and the VWF:Ag/ADAMTS13:AC ratio levels were significantly lower in those with SD, PR, and CR than those with PD ($P < 0.05$ for both) (Figure 1B and C). ADAMTS13:AC level was directly correlated with albumin ($r = 0.457$, $P < 0.05$), and VWF:Ag and the VWF:Ag/ADAMTS13:AC ratio levels were directly correlated with total bilirubin($r = 0.329$, $P < 0.05$ and $r = 0.316$, $P < 0.05$, respectively). In addition, the patients were categorized into two, according to the TNM stage (TNM stage 2 and 3 is 21 patients, and TNM stage 4 is 20 patients). The VWF:Ag/ADAMTS13:AC ratio levels was higher in patients with TNM stage 4 HCC than those with TNM stage 2 and 3 HCC (Figure 2).

Predictive factors for sorafenib response

ADAMTS13:AC and the VWF:Ag/ADAMTS13:AC ratio were associated with sorafenib response in univariate analysis (Table 2). To determine predictive factors for sorafenib response, we performed multivariate analysis using albumin, DCP, VEGF, maximum tumor size, distant metastasis, ADAMTS13:AC, VWF:Ag, and the VWF:Ag/ADAMTS13:AC ratio, which had *P*-values of < 0.2 in the univariate analysis. The VWF:Ag/ADAMTS13:AC ratio was significantly associated with sorafenib response in multivariate analysis (Table 2). The receiver operating characteristic (ROC) analysis revealed that a cutoff VWF:Ag/ADAMTS13:AC ratio of 2.609 had a specificity of 84.2% and a sensitivity of 88.2%, and the area under the ROC curve (AUC) was 0.836 (Figure 3). Next, the study patients were categorized into two groups according to the ROC cutoff VWF:Ag/ADAMTS13:AC ratio: Low (VWF:Ag/ADAMTS13:AC ratio < 2.609) and high (VWF:Ag/ADAMTS13:AC ratio ≥ 2.609). The patients with a high VWF:Ag/ADAMTS13:AC ratio had significantly higher VEGF levels than those with a low VWF:Ag/ADAMTS13:AC ratio (Figure 4A), indicating that the VWF:Ag/ADAMTS13:AC ratio might be associated with VEGF in patients with HCC. However, the patients with VEGFR-2, AFP, DCP and AFP-L3%

Table 1 Characteristics of patients with hepatocelullar carcinoma receiving sorafenib treatment according to treatment outcomes

Variable	Total(*n* = 41)	SD + PR + CR(*n* = 17)	PD(*n* = 24)	*P* value
Age (yr)	74.0 (69.0-81.0)	74.0 (71.0-78.0)	74.0 (63.5-70.0)	0.400
Sex (male/female)	38/3	17/0	21/3	0.254
Etiology (HBV/HCV/NASH/alcohol)	7/20/3/11	0/13/1/3	7/7/2/8	0.0707
Albumin (g/dL)	3.6 (3.3-3.9)	3.7 (3.5-4.0)	3.5 (3.3-3.7)	0.0827
Prothrombin time (%)	82.0 (77.0-89.0)	83.0 (78.0-89.0)	82.0 (78.5-91.0)	0.751
Total bilirubin (mg/dL)	0.8 (0.6-0.9)	0.7 (0.5-0.9)	0.8 (0.6-0.9)	0.397
Platelet count ($\times 10^4/mm^3$)	14.1 (11.1-17.3)	16.1 (9.4-18.3)	14.1 (11.6-16.9)	0.568
AFP (ng/mL)	121.8 (11.3-2611.0)	31.8 (6.7-161.0)	286.0 (41.6-5193.5)	0.103
DCP (mAU/mL)	359.5 (58.0-5277.5)	192 (24.5-5177)	5177 (183-17214)	0.012
AFP-L3% (%)	13.2 (1.7-42.4)	12.3 (1.45-34.3)	21.8 (1.85-47.7)	0.529
VEGF (pg/mL)	25.8 (14.1-40.1)	18.5 (10.0-35.1)	28.2 (22.0-50.7)	0.106
VEGFR-2 (pg/mL)	6500 (5750-7400)	6400 (5200-7100)	6800 (6350-7600)	0.211
Maximum tumor size (cm)	3.3 (2.5-7.7)	3.2 (2.0-6.0)	4.3 (2.7-10.8)	0.220
Tumor number (1/2/3/4 or more/only distant metastasis)	3/2/1/33/2	0/2/1/13/1	3/0/0/20/1	0.131
PVTT (presence/absence)	7/34	2/15	5/19	0.679
Distant metastasis (presence/absence)	17/24	10/7	7/17	0.107
Child-pugh score	5 (5-6)	5 (5-6)	5 (5-6)	0.469
UICC TNM stage (2/3/4)	5/16/20	1/6/10	4/10/10	0.455
Observation (survival) period (d)	328 (156-530)	564 (405-880)	162 (116-319)	0.000322

Data are expressed as median (Interquartile range). *P*-values represent comparisons between patients with HCC who had SD+PR+CR and PD. HCC: Hepatocelullar carcinoma; HCV: Hepatitis C virus; HBV: Hepatitis B virus; NASH: Non-alcoholic steatohepatitis; AFP: Alpha fetoprotein; DCP: Des-γ-carboxy prothrombin; AFP-L3%: *Lens culinaris* agglutinin-reactive alpha-fetoprotein; VEGF: Vascular endothelial growth factor; VEGFR-2: VEGF receptor-2; PVTT: Portal vein tumor thrombosis; UICC: The Union for the International Cancer Control; TNM stage: Tumor-node-metastasis stage; SD: Stable disease; PR: Partial response; CR: Complete response; PD: Progressive disease.

levels were not different between the low and high VWF:Ag/ADAMTS13:AC ratio groups.

Prognostic factors for patients with HCC receiving sorafenib treatment

In univariate analysis, age, DCP, and ADAMTS13:AC were associated with prognosis in patients with HCC receiving sorafenib treatment (Table 3). To determine prognostic factors in patients with HCC receiving sorafenib treatment, we performed multivariate analysis using age, sex, DCP, tumor number, ADAMTS13:AC, and the VWF:Ag/ADAMTS13:AC ratio, which had *P*-values < 0.2 in the univariate analysis. ADAMTS13:AC was associated significantly with prognosis in multivariate analysis (Table 3). Therefore, the patients were categorized into two groups according to the median cutoff ADAMTS13:AC: Low (ADAMTS13:AC < 78.0) and high (ADAMTS13:AC ≥ 78.0). The patients with a high ADAMTS13:AC had significantly longer PFS and OS than those with a low ADAMTS13:AC (Figure 5). The patients with a low ADAMTS13:AC had significantly higher VEGF levels than those with a high ADAMTS13:AC (Figure 4B). These results indicated that ADAMTS13:AC might be associated with VEGF in patients with HCC. However, the patients with VEGFR-2, AFP, DCP, and AFP-L3% levels were not different between the low and high ADAMTS13:AC groups.

DISCUSSION

The results of the present study suggest that the VWF:Ag/ADAMTS13:AC ratio and ADAMTS13:AC are potential biomarkers for sorafenib response and prognosis, respectively, in patients with HCC receiving sorafenib treatment. Previous studies reported that Child-Pugh score A patients with HCC receiving sorafenib treatment had longer OS and PFS than those with Child-Pugh score B patients[29] and that VWF:Ag was associated with prognosis in cirrhotic patients[30]. Furthermore, we previously reported that ADAMTS13:AC and VWF:Ag were associated with functional liver capacity[12,13] and that ADAMTS13:AC was associated with prognosis in cirrhotic patients[6]. In other words, ADAMTS13:AC and VWF:Ag are useful biomarkers to evaluate functional liver capacity in detail for cirrhotic patients[6,30]. As

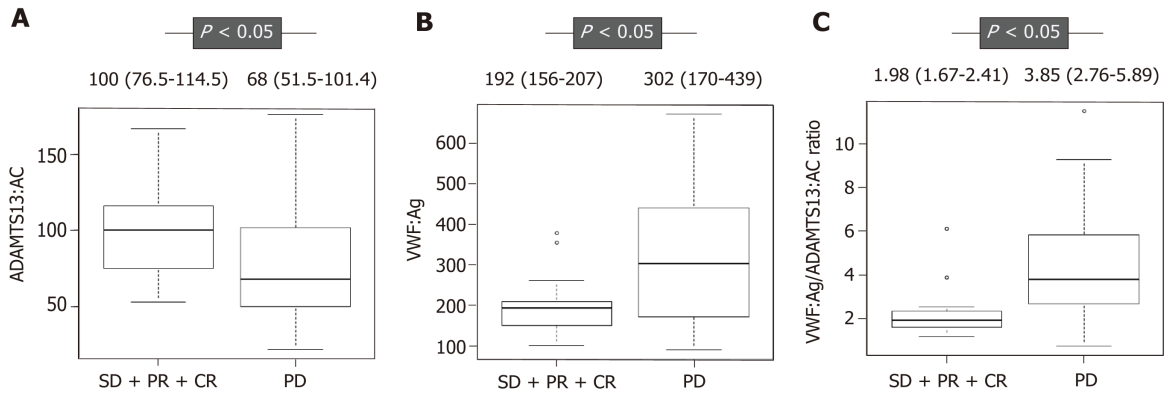

Figure 1 Plasma ADAMTS13:AC and VWF:Ag levels in patients with hepatocellular carcinoma receiving sorafenib treatment. A: ADAMTS13:AC level was significantly higher in patients with hepatocellular carcinoma receiving sorafenib treatment who achieved stable disease (SD), partial response (PR), and complete response (CR) than those who achieved progressive disease (PD) ($P < 0.05$); B, C: In contrast, VWF:Ag, and the VWF:Ag/ADAMTS13:AC ratio levels were significantly lower in those with SD, PR, and CR than those with PD ($P < 0.05$, $P < 0.05$). ADAMTS13: A disintegrin-like and metalloproteinase with thrombospondin type 1 motifs 13; ADAMTS13:AC: ADAMTS13 activity; VWF: Von Willebrand factor; VWF:Ag: VWF antigen; VWF:Ag/ADAMTS13:AC ratio: The ratio of VWF:Ag to ADAMTS13:AC; SD: Stable disease; PR: Partial response; CR: Complete response; PD: Progressive disease.

the result, the VWF:Ag/ADAMTS13:AC ratio and ADAMTS13:AC might be associated with sorafenib response and prognosis, respectively, in patients with HCC receiving sorafenib treatment.

A recent study reported that HCC patients with Barcelona Clinic Liver Cancer (BCLC) stage B receiving sorafenib treatment had longer OS than those with BCLC stage C[29]. The current study revealed that the VWF:Ag/ADAMTS13:AC ratio was associated with TNM stage in HCC. Furthermore, several studies reported that the VWF:Ag/ADAMTS13:AC ratio was associated with TNM stage and prognosis in various cancers[24,31,32]. The association of the VWF:Ag/ADAMTS13:AC ratio and ADAMTS13:AC with sorafenib response and prognosis, respectively, in patients with HCC receiving sorafenib treatment reflects that the ADAMTS13–VWF imbalance might be associated with tumor stage. Furthermore, this may be caused by blood coagulation cascade associated with cancer development[21,22] because the ADAMTS13–VWF imbalance associated with blood coagulation cascade[11].

In addition, angiogenesis plays an important role in LC and HCC development, which are related to VEGF, as VEGF levels are increased in patients with LC and HCC[18-20]. Recent studies reported that VWF reduced VEGF-depend angiogenesis via multiple intracellular and extracellular pathways involving integrin avβ3 and angiopoietin-2[14-16] and that ADAMTS13 induced angiogenesis by ADAMTS13-mediated cleavage of VWF and ADAMTS13-mediated VEGFR-2 phosphorylation, which lead to enhanced VEGF expression[17]. Randi *et al*[16] revealed the critical role of the balance between ADAMTS13 and VWF in regulating blood vessel formation. Furthermore, we observed that VWF:Ag was a predictive factor[8] and that the VWF:Ag/ADAMTS13:AC ratio was a diagnostic factor (unpublished observations) for HCC in cirrhotic patients. As sorafenib inhibits VEGF and the change in VEGF during sorafenib treatment is associated with the prognosis of patients with HCC receiving sorafenib treatment[4], the VWF:Ag/ADAMTS13:AC ratio and ADAMTS13:AC might be associated sorafenib response and prognosis, respectively, in patients with HCC receiving sorafenib treatment, through angiogenesis. In fact, in the present study, we also found that the VWF:Ag/ADAMTS13:AC ratio and ADAMTS13:AC were associated with VEGF levels.

Furthermore, antiplatelet therapy inhibits PDGF, which induces HCC development[33]. Recent studies reported that antiplatelet therapy prevented HCC development in cirrhotic patients[34] and improved survival in a mouse model of chronic hepatitis B[33]. An imbalance between ADAMTS13 and VWF induces platelet thrombus formation[11] and thereby is possibly associated with PDGF. The association of the VWF:Ag/ADAMTS13:AC ratio and ADAMTS13:AC with sorafenib response and prognosis in patients with HCC receiving sorafenib treatment might be occurring via PDGF, which is inhibited by sorafenib and VEGF. The relationship of the ADAMTS13-VWF imbalance with angiogenic factors requires further investigation.

There are several promising candidate biomarkers for predicting sorafenib response and prognosis in patients with HCC receiving sorafenib treatment, including VEGF-A, angiopoietin-2, insulin-like growth factor-1, and neutrophil/lymphocyte ratio[29,35], most of which are not identified as biomarkers for these patients due to high

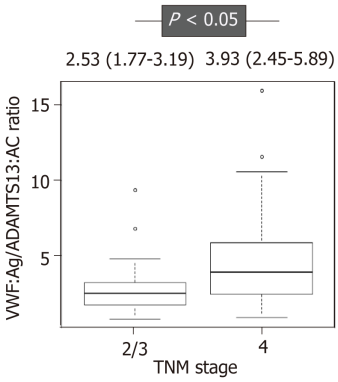

Figure 2 The relationship between the VWF:Ag/ADAMTS13:AC ratio and tumor-node-metastasis stage. The VWF:Ag/ADAMTS13:AC ratio level was significantly higher in patients with tumor-node-metastasis stage 4 hepatocellular carcinoma (HCC) than those with stage 2 and 3 HCC. ADAMTS13: A disintegrin-like and metalloproteinase with thrombospondin type 1 motifs 13; ADAMTS13:AC: ADAMTS13 activity; VWF: Von Willebrand factor; VWF:Ag: VWF antigen; VWF:Ag/ADAMTS13:AC ratio: The ratio of VWF:Ag to ADAMTS13:AC; TNM stage: Tumor-node-metastasis stage.

costs and limited practicality in a clinical setting[29,35]. In addition, while these biomarkers were reported as prognostic factors for OS in patients with HCC receiving sorafenib treatment, their relationship with PFS was not determined. Importantly, OS is affected by after-treatment. In fact, in the present study, several patients underwent TACE and/or hepatic arterial infusion chemotherapy using cisplatin. Therefore, we propose that PFS might be more important than OS for evaluating sorafenib treatment efficiency in patients with HCC in clinical setting. Because ADAMTS13:AC is a prognostic factor for OS and PFS in patients with HCC receiving sorafenib treatment, we believe that ADAMTS13:AC is a more useful biomarker than other biomarkers.

The present study has several limitations, including a short observation period and the small sample size. Cirrhotic patients with HCC occasionally develop thrombosis or inflammation, including portal thrombosis and bacterial overgrowth and translocation, which may impact the VWF:Ag/ADAMTS13:AC ratio and ADAMTS13:AC and impact their value as biomarkers. In addition, only 7.3% of patients were female (male: 38, female: 3). We believe that the difference in gender had no effects in our study because a previous study has reported that the relationships between ADAMTS13:AC and other parameters (*e.g.*, albumin, total bilirubin, aspartate aminotransferase, alkaline phosphatase, and creatinine) are not associated with gender bias[36].

In summary, the VWF:Ag/ADAMTS13:AC ratio and ADAMTS13:AC, which were associated with VEGF, were independent predictive factors for sorafenib response and prognosis, respectively, in patients with HCC receiving sorafenib treatment. To our knowledge, this is the first report the ADAMTS13-VWF imbalance in association with sorafenib response and prognosis in patients with HCC receiving sorafenib treatment.

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This work was completed with the help of Ms. Yoshie Nakai, Professor Hiroshi Fukui, and Professor Masahito Uemura.

Table 2 Predictive factors for sorafenib response

Univariate analysis	OR (95%CI)	*P* value
Age (per 1 yr increase)	1.05 (0.967-1.13)	0.2660
Sex (male *vs* female)	0.556 (0.0464-6.66)	0.9950
Viral hepatitis (presence *vs* absence)	0.527(0.123-2.27)	0.6430
Albumin (per 1 g/dL decrease)	6.59 (0.827-52.6)	0.0750
Prothrombin time (per 1% decrease)	0.977 (0.928-1.03)	0.3910
Total bilirubin (per 1 mg/dL increase)	0.376 (0.0378-3.74)	0.4040
Platelet count (per 10^4/μL decrease)	1.040 (0.937-1.14)	0.4970
AFP (per 1 ng/mL increase)	1.000 (1.000-1.00)	0.5330
DCP (per 1 mAU/mL increase)	1.00 (0.999-1.00)	0.1100
AFP-L3% (per 1% increase)	1.00 (0.996-1.01)	0.4420
VEGF (per 1 pg/mL increase)	0.98 (0.949-1.01)	0.1950
VEGFR-2 (per 1 pg/mL increase)	1.00 (0.999-1.000)	0.2210
Maximum tumor size (per 1 cm increase)	0.892 (0.752-1.06)	0.1900
Tumor number (per 1 increase)	1.11 (0.646-1.90)	0.7070
PVTT (presence *vs* absence)	0.835 (0.455-1.53)	0.5590
Distant metastasis (presence *vs* absence)	2.450 (0.64-9.37)	0.1910
ADAMTS13:AC (per 1% increase)	1.020 (1.0001-1.050)	0.0039
VWF:Ag (per 1% increase)	0.996 (0.991-1.000)	0.0740
VWF:Ag/ADAMTS13:AC (per 1 increase)	0.465 (0.265-0.817)	0.0077
Multivariate analysis		
VWF:Ag/ADAMTS13:AC (per 1 increase)	0.495 (0.281-0.870)	0.0147

AFP: Alpha fetoprotein; DCP: Des-γ-carboxy prothrombin; AFP-L3%: *Lens culinaris* agglutinin-reactive alpha-fetoprotein; VEGF: Vascular endothelial growth factor; VEGFR-2: VEGF receptor-2; PVTT: Portal vein tumor thrombosis; ADAMTS13: A disintegrin-like and metalloproteinase with thrombospondin type 1 motifs 13; ADAMTS13:AC: ADAMTS13 activity; VWF: Von Willebrand factor; VWF: Ag: VWF antigen; VWF:Ag/ADAMTS13:AC ratio: The ratio of VWF:Ag to ADAMTS13:AC; OR: Odds ratio; CI: Confidence interval.

Table 3 Prognostic factors for patients with hepatocellular carcinoma receiving sorafenib treatment

Univariate analysis	HR (95%CI)	*P* value
Age (per 1 yr increase)	1.16 (1.032-1.304)	0.0127
Sex (male *vs* female)	0.062 (0.000382-1.008)	0.0561
Viral hepatitis (presence *vs* absence)	1.416(0.405-4.95)	0.5862
Albumin (per 1 g/dL decrease)	0.427 (0.0750-2.44)	0.3388
Prothrombin time (per 1% decrease)	1.008 (0.969-1.048)	0.3910
Total bilirubin (per 1 mg/dL increase)	4.203 (0.417-42.30)	0.2230
Platelet count (per 10^4/μL decrease)	0.906 (0.807-1.018)	0.4970
AFP (per 1 ng/mL increase)	1.000 (1.000-1.00)	0.3334
DCP (per 1 mAU/mL increase)	1.000 (1.000-1.00)	0.0136
AFP-L3% (per 1% increase)	0.999 (0.969-1.031)	0.9484
VEGF (per 1 pg/mL increase)	0.982 (0.952-1.01)	0.2426
VEGFR-2 (per 1 pg/mL increase)	1.00 (0.999-1.000)	0.8947
Maximum tumor size (per 1 cm increase)	1.026 (0.868-1.214)	0.7622
Tumor number (per 1 increase)	0.751 (0.5-1.129)	0.1690
PVTT (presence *vs* absence)	0.347 (0.0384-3.141)	0.3464
Distant metastasis (presence *vs* absence)	0.489 (0.129-1.854)	0.2936
ADAMTS13:AC (per 1% increase)	0.936 (0.895-0.978)	0.0035
VWF:Ag (per 1% increase)	0.996 (0.991-1.000)	0.9227
VWF:Ag/ADAMTS13:AC (per 1 increase)	1.33 (0.998-1.772)	0.0520
Multivariate analysis		
ADAMTS13:AC (per 1% increase)	0.937 (0.895-0.980)	0.0045

HCC: Hepatocellular carcinoma; AFP: Alpha fetoprotein; DCP: Des-γ-carboxy prothrombin; AFP-L3%: *Lens culinaris* agglutinin-reactive alpha-fetoprotein;

VEGF: Vascular endothelial growth factor; VEGFR-2: VEGF receptor-2; PVTT: Portal vein tumor thrombosis; ADAMTS13: A disintegrin-like and metalloproteinase with thrombospondin type 1 motifs 13; ADAMTS13:AC: ADAMTS13 activity; VWF: Von Willebrand factor; VWF:Ag: VWF antigen; VWF:Ag/ADAMTS13:AC ratio: The ratio of VWF:Ag to ADAMTS13:AC; HR: Hazard ratio; CI: Confidence interval.

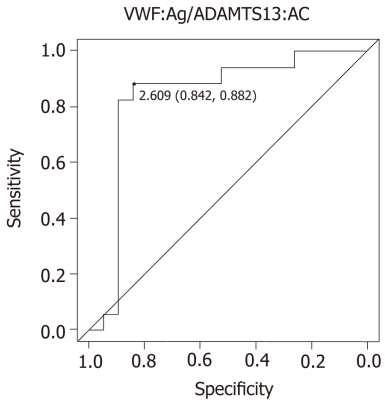

Figure 3 Diagnostic accuracy of the VWF:Ag/ADAMTS13:AC for sorafenib response. The receiver operating characteristic analysis of the VWF:Ag/ADAMTS13:AC ratio for sorafenib response reveals that a cutoff VWF:Ag/ADAMTS13:AC ratio of 2.609 has a specificity of 84.2% and a sensitivity of 88.2%, with an area under the receiver operating characteristic curve of 0.836. ADAMTS13: A disintegrin-like and metalloproteinase with thrombospondin type 1 motifs 13; ADAMTS13:AC: ADAMTS13 activity; VWF: Von Willebrand factor; VWF:Ag: VWF antigen; VWF:Ag/ADAMTS13:AC ratio: The ratio of VWF:Ag to ADAMTS13:AC.

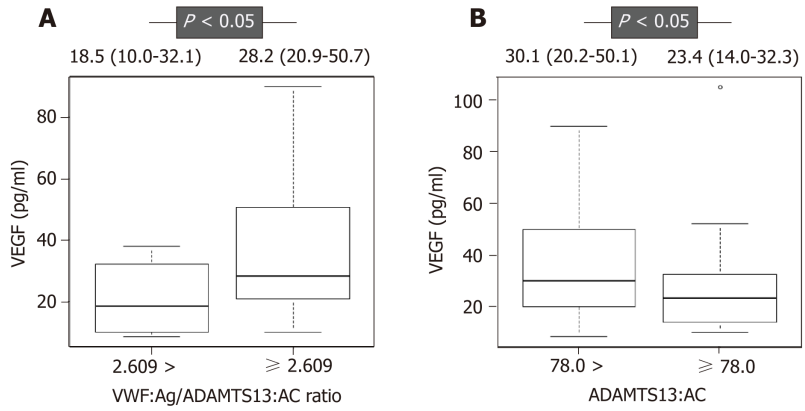

Figure 4 Vascular endothelial growth factor is associated with the VWF:Ag/ADAMTS13:AC ratio and ADAMTS13:AC in patients with hepatocellular carcinoma receiving sorafenib treatment. A: Hepatocellular carcinoma (HCC) patients with a VWF:Ag/ADAMTS13:AC ratio ≥ 2.609 had significantly higher vascular endothelial growth factor (VEGF) levels than those with a VWF:Ag/ADAMTS13:AC ratio < 2.609; B: HCC patients with an ADAMTS13:AC < 78.0 had significantly higher VEGF levels than those with an ADAMTS13:AC ≥ 78.0. ADAMTS13: A disintegrin-like and metalloproteinase with thrombospondin type 1 motifs 13; ADAMTS13:AC: ADAMTS13 activity; VWF: Von Willebrand factor; VWF:Ag: VWF antigen; VWF:Ag/ADAMTS13:AC ratio: The ratio of VWF:Ag to ADAMTS13:AC; VEGF: Vascular endothelial growth factor.

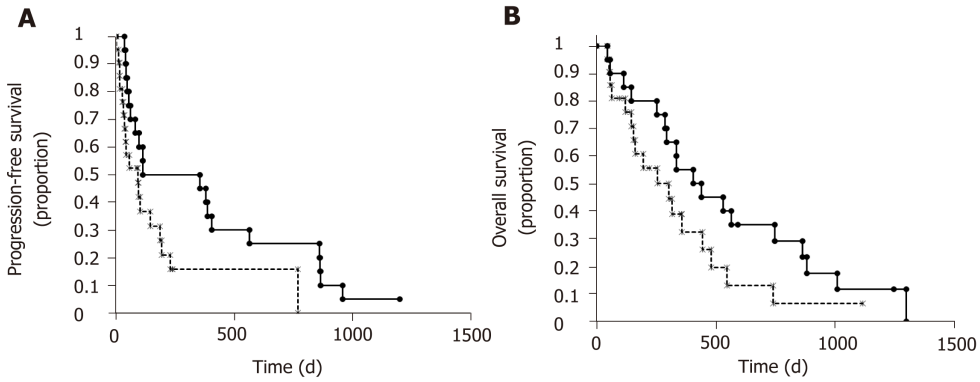

Figure 5 ADAMTS13 is associated with progression-free survival and overall survival in patients with hepatocellular carcinoma receiving sorafenib treatment. Hepatocellular carcinoma (HCC) patients with an ADAMTS13:AC ≥ 78.0 had significantly longer progression-free survival (A) and overall survival (B) than those with an ADAMTS13:AC < 78.0. Solid and dotted lines indicate HCC patients with an ADAMTS13:AC ≥ 78.0 and an ADAMTS13:AC < 78.0, respectively. ADAMTS13: A disintegrin-like and metalloproteinase with thrombospondin type 1 motifs 13; ADAMTS13:AC: ADAMTS13 activity.

ARTICLE HIGHLIGHTS

Research background

Sorafenib reportedly improves overall survival (OS) significantly in patients with HCC. Prediction of sorafenib response and prognosis in patients with HCC receiving sorafenib treatment are important due to the potentially serious side effects of sorafenib.

Research motivation

A disintegrin-like and metalloproteinase with thrombospondin type-1 motifs 13 (ADAMTS13) and von Willebrand factor (VWF) are associated with the pathophysiology of liver cirrhosis and HCC through their roles in hypercoagulability; they are also associated with angiogenesis via vascular endothelial growth factor (VEGF). The imbalance between ADAMTS13 and VWF was associated with prognosis of various cancers in patients undergoing chemotherapy.

Research objectives

To investigate ADAMTS13 and VWF as potential biomarkers for sorafenib response and prognosis in patients with HCC receiving sorafenib treatment.

Research methods

Forty-one patients with HCC receiving sorafenib treatment were included in this study. The initial daily sorafenib dose was 400 mg in all patients. ADAMTS13 activity (ADAMTS13:AC), VWF antigen (VWF:Ag), VEGF levels were determined by enzyme-linked immunosorbent assay. Univariate and multivariate analyses were used to determine predictive factors for sorafenib response and prognosis in patients with HCC receiving sorafenib treatment.

Research results

Multivariate analysis showed that the VWF:Ag/ADAMTS13:AC ratio was the only predictive factor for sorafenib response and ADAMTS13:AC was the only prognostic factor in patients with HCC receiving sorafenib treatment. The patients with a low ADAMTS13:AC (< 78.0) had significantly higher VEGF levels than those with a high ADAMTS13:AC (≥ 78.0) ($P < 0.05$).

Research conclusions

The VWF:Ag/ADAMTS13:AC ratio and ADAMTS13:AC are potentially useful biomarkers for sorafenib response and prognosis, respectively, in patients with HCC receiving sorafenib treatment.

Research perspectives

This is the first report the ADAMTS13-VWF imbalance in association with sorafenib response and prognosis in patients with HCC receiving sorafenib treatment.

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ORIGINAL ARTICLE

Observational Study

Analysis of B-ultrasound and contrast-enhanced ultrasound characteristics of different hepatic neuroendocrine neoplasm

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Abstract

BACKGROUND

Hepatic neuroendocrine neoplasm (hNEN) is a highly heterogeneous tumor. The exact identification of the source and malignant degree of hNEN is important. However, there is a lack of information regarding diagnosis of hNEN with imaging. In addition, no studies have compared the imaging between hNEN and hepatocellular carcinoma (HCC) and among different sources and malignant degrees of hNEN.

AIM

To compare the ultrasound characteristics between hNEN and HCC and among different sources and malignant degrees of hNEN.

METHODS

A total of 55 patients with hNEN were recruited and defined as the hNEN group. Among them, 35 cases of hNET were defined as the hNET group. Twenty cases of hepatic neuroendocrine carcinoma (hNEC) were defined as the hNEC group. Among the 55 lesions, 29 were transferred from the pancreas, 20 were from the gastrointestinal tract, and six were from other sites. In total, 55 patients with HCC were recruited and defined as the HCC group. The characteristic differences of B-mode ultrasound and contrast-enhanced ultrasound (CEUS) between hNEN and HCC and among different sources and malignant degrees of hNEN were compared.

RESULTS

In the hNEN group, the proportions of multiple liver lesions, unclear borders,

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and high echo lesions were higher than those in the HCC group. The proportions of non-uniform echo and peripheral acoustic halo were lower than those in the HCC group ($P < 0.05$). The washout to iso-enhancement time and washout to hypo-enhancement time were lower than those in the HCC group ($P < 0.05$). The characteristics of B-ultrasound and CEUS among different sources of hNEN were similar, and the differences were not statistically significant ($P > 0.05$). B-mode ultrasound characteristics of hNET and hNEC were similar. The proportions of low enhancement at portal venous phase, non-uniform enhancement forms, and combined tumor vasculature in the hNEC group were larger than those in the hNEN group ($P < 0.05$).

CONCLUSION
Compared with HCC, hNEN showed multiple intrahepatic lesions, uniform high echo, uniform high enhancement at arterial phase, and rapid washout. Low enhancement at portal venous phase, overall non-uniform enhancement form, and the proportion of combined tumor vasculature in hNEC were larger than those in hNET.

Key words: Hepatic neuroendocrine neoplasm; Hepatic neuroendocrine tumor; Hepatic neuroendocrine carcinoma; B-ultrasound; Contrast-enhanced ultrasound

Core tip: Clinically, hepatic neuroendocrine neoplasm (hNEN) is rare, and few reports are currently available on the imaging diagnosis of hNENs. In this study, by comparing hNEN and hepatocellular carcinoma, hNEN from different sources, and differentiation, it was found that the ultrasound characteristics of hNEN are mostly multiple, uniform hyperechoic masses. The enhancement at the arterial phase was mostly uniform and high, and the washout was rapid compared with hepatocellular carcinoma. Compared with hepatic neuroendocrine tumor, the enhancement at the portal venous phase of hepatic neuroendocrine carcinoma was low, and the enhancement form was non-uniform.

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INTRODUCTION

Neuroendocrine neoplasm (NEN) is a highly heterogeneous tumor. The liver is the most important metastatic part of NEN, which is mostly transferred from other organs, such as the gastrointestinal tract. Therefore, hepatic NEN (hNEN) is more common than primary hNEN[1-3]. The manifestations of hNEN patients are complex and mostly non-specific. Patients with hNEN often present with liver discomfort and bloating. It is necessary to identify hNEN and hepatocellular carcinoma (HCC). In addition, all NENs have malignant potential. NEN from different sources and malignant degrees differ greatly in outcome and treatment. Hence, it is important to identify accurately the source and malignant degree of hNEN.

Currently, the diagnosis of hNEN mainly depends on the results of pathological examination and immunohistochemistry[4-6]. Although pathological examination and immunohistochemistry are the gold standard for diagnosis, they are invasive examinations. They can only be used as a means of verification and cannot be used as a screening tool for diseases. Clinically, initial screening is required through non-invasive examinations (*e.g.*, imaging examinations, laboratory examinations, *etc*), and pathological diagnosis is performed on highly suspected patients. However, due to the rareness of hNEN, there is a lack of current information regarding imaging examinations, and there is little experience in identifying hNEN and HCC, hNEN from different sources, and malignant degrees.

Beard *et al*[7] reported that hNEN and HCC had similarities in ultrasound

performance, which may cause misdiagnosis due to insufficient understanding. Some studies compared the hNEN characteristics of different sources and malignant degrees and found that the B-ultrasound and contrast-enhanced ultrasound (CEUS) performance of hNEN from different sources and malignant degrees were different[8-10]. These findings suggest that we can identify hNEN by ultrasound and CEUS, but its clinical application value has not been confirmed. Therefore, the present study compared the ultrasound performance between hNEN and HCC. In addition, the characteristics of B-mode ultrasound and CEUS from different sources and malignant degrees of hNEN were analyzed in order to provide a reference for the diagnosis and treatment of hNEN.

MATERIALS AND METHODS

Research object

A total of 55 patients with hNEN admitted to Cangzhou Central Hospital from January 2014 to May 2018 were recruited. All patients obtained a complete B-mode ultrasound and CEUS data. They were defined as the hNEN group. Among them, 27 were males and 28 were females with an age range of 36-68 years old and an average of 55.23 ± 14.52 years old. Three patients in the hNEN group had hepatitis. The inclusion criteria of hNEN were: Surgical resection or biopsy was confirmed as hNEN, and immunohistochemistry confirmed that ChrA or Syno was positive. The exclusion criteria were: HCC, mixed liver cancer, hilar cholangiocarcinoma, and extrahepatic cholangiocarcinoma. According to the World Health Organization classification of the digestive system tumor (2010) neuroendocrine tumor (NET) grading standard[11], 35 cases of hepatic NET (hNET) (G1 and G2) were defined as the hNET group, and 20 cases of hNEC (G3) were defined as the hNEC group. Among the 55 hNEN lesions, 29 were transferred from the pancreas, 20 were from the gastrointestinal tract, and six were from other sites (two cases from the gallbladder, two cases from the abdomen, and two cases from the lung). During the study period, 55 patients with HCC were recruited as the HCC group. There were 38 males and 17 females with an age range of 35-71 years old and an average age of 54.29 ± 17.27 years old. There were 51 HCC patients associated with hepatitis, and the hepatitis infection rate was significantly higher than that of hNEN patients. The difference was statistically significant (χ^2 = 86.443, P = 0.000). All patients signed informed consent, and this study was reviewed by the Ethics Committee of Cangzhou Central Hospital.

Research methods

Ultrasound examination: Ultrasound examination was performed using a Philips ultrasound affinity 70 diagnostic instruments equipped with CEUS imaging software. B-mode ultrasound and CEUS examinations were performed in each patient. The patient was placed in a supine position. The depth, focus, gain, and grayscale and color Doppler (CDFI) range were adjusted before examination. B-mode ultrasound examinations, including CDFI scans, were performed first. Lesion diameter (unit: cm), number (single/multiple), lesion property (solid/cyst), echo uniformity (uniform/non-uniform), echo level (high/low/mixed/equal), boundary (clear/unclear), accompanying signs (peripheral acoustic halo, posterior echo attenuation) of the liver lesions, and CDFI images were recorded.

Subsequently, a 2.4 mL contrast agent of SonoVue (Bracco) was used for CEUS. After the bolus injection into the left median cubital vein, 5 mL of saline was injected. The timing was started when the injection began. The whole examination process was about 3-5 min, and the image data were recorded. The time phase of hepatic CEUS was: 10-30 s after the injection of the contrast agent was the arterial phase, 31-120 s was the portal venous phase, and 121-360 s was the late phase. All examinations were performed by physicians with more than 10 years of ultrasound experience in our hospital.

Data collection and image analysis: B-mode ultrasound lesion diameter, number, boundary, lesion property, echo level, echo uniformity, and the number and proportion of accompanying signs (peripheral acoustic halo, posterior echo attenuation) of the liver lesions were observed and recorded. The characteristic differences of B-mode ultrasound between hNEN and HCC groups, transferred from different hNEN sources, and between hNEC and hNET groups were compared.

CEUS: The initial enhancement time (unit:s) of liver parenchyma and lesions was recorded. The washout to iso-enhancement time (unit:s) and washout to hypo-enhancement time (unit:s) of liver lesions were recorded as well. Then, the number and proportion of different enhancement levels at arterial phase (reference to the

enhancement level of adjacent liver tissue, divided into high/equal/low enhancement), enhancement levels at portal venous phase and late phase (equal/low enhancement), enhancement forms (uniform or non-uniform enhancement), enhancement-washout modes (fast enhancement and washout/equal enhancement and fast washout/low enhancement and fast washout), and special signs (adjacent and internal tumor vasculature, tumor necrosis no-enhancement zone, capsule enhancement in the late phase) of liver lesions were recorded. The characteristic differences of CEUS between hNEN and HCC groups, transferred from different hNEN sources, and between hNEC and hNET groups were compared.

Statistical analysis

All statistical analyses were performed with SPSS version 19.0 (IBM, Armonk, NY, United States) software. The numerical data were expressed as mean ± SD and the categorical variables as number and percentage. The *t* test was used to compare the two groups of numerical data, and the three groups of numerical data were compared using one-way analysis of variance. The comparisons between the categorical variables were performed by chi-square test. If the minimum theoretical frequency was less than one, the Fisher's exact test was used. $P < 0.05$ was considered a statistically significant difference.

RESULTS

Pathological features of hNEN

Hematoxylin-eosin staining showed that the tumors were arranged by uniform circular or oval cells, which were nested or glandularly distributed. The cells were well-differentiated. There were fewer mitotic figures, and the atypia was not obvious (Figure 1). Immunohistochemical staining showed that 42 patients with hNEN were positive for ChrA (Figure 2), and 45 patients were positive for Syno (Figure 3).

Comparison of B-mode ultrasound characteristics between the hNEN group and the HCC group

Among all the B-mode ultrasound features, lesion diameter and the proportions of different lesion property and posterior echo attenuation were similar between the hNEN and HCC groups, and the differences were not statistically significant ($P > 0.05$). The proportions of multiple liver lesions, unclear boundary, and high echo lesion in the hNEN group were higher than those in the HCC group, and the differences were statistically significant ($P < 0.05$). The proportions of non-uniform echo and peripheral acoustic halo in the hNEN group were lower than those in the HCC group, and the differences were statistically significant ($P < 0.05$; Table 1).

Comparison of CEUS characteristics between hNEN and HCC groups

The initial enhancement time was similar in the hNEN and HCC groups, and the difference was not statistically significant ($P > 0.05$). The washout to iso-enhancement time and washout to hypo-enhancement time in the hNEN group were lower than those in the HCC group. The differences were statistically significant ($P < 0.05$).

The proportions of different CEUS enhancement characteristics, including enhancement at arterial phase, portal venous phase, and late phase, enhancement-washout mode, enhancement form, tumor vasculature, tumor necrosis, and capsule enhancement were similar in the two groups, and the differences were not statistically significant ($P > 0.05$; Table 2).

Comparison of B-mode ultrasound characteristics transferred from different sources of hNEN

The lesion diameter in hNEN lesions transferred from the gastrointestinal tract, pancreas, and other sites was similar, and there was no statistical significance ($P > 0.05$). In addition, the proportions of hNEN B-mode ultrasound characteristics, including number of liver lesions, lesion property, boundary, echo level, echo uniformity, posterior echo attenuation, and peripheral acoustic halo, transferred from different sources were similar, and the differences were not statistically significant ($P > 0.05$; Table 3).

Comparison of CEUS characteristics transferred from different sources of hNEN

The initial enhancement time, washout to iso-enhancement time, and washout to hypo-enhancement time of hNEN transferred from the gastrointestinal tract, pancreas, and other sites were similar. The differences were not statistically significant ($P > 0.05$). The proportions of CEUS enhancement characteristics transferred from

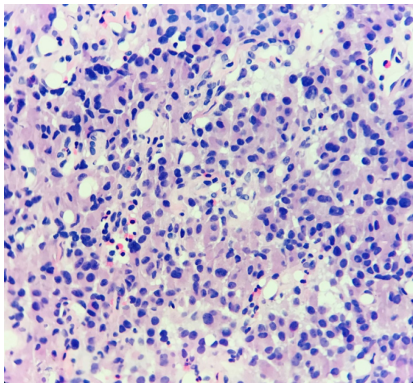

Figure 1 Hematoxylin-eosin staining results of hepatic neuroendocrine neoplasm.

different sources of hNEN, including enhancement at arterial phase, portal venous phase and late phase, enhancement-washout mode, enhancement form, tumor vasculature, tumor necrosis, and capsule enhancement, were similar, and the differences were not statistically significant ($P > 0.05$; Table 4).

Comparison of B-mode ultrasound characteristics between hNET and hNEC groups

The difference in lesion diameter between hNET and hNEC groups was not statistically significant ($P > 0.05$). The proportions of B-mode ultrasound features, including number of liver lesions, lesion property, boundary, echo level, echo uniformity, posterior echo attenuation, and peripheral acoustic halo, between hNET and hNEC groups were similar, and the differences were not statistically significant ($P > 0.05$; Table 5).

Comparison of CEUS characteristics between hNET and hNEC groups

There was no significant difference between hNEN and hNEC groups in terms of initial enhancement time, washout to iso-enhancement time, and washout to hypo-enhancement time ($P > 0.05$). Among the CEUS enhancement characteristics, the proportions of low enhancement at portal venous phase, non-uniform enhancement forms, and no tumor vasculature in the hNEC group were greater than those in the hNEN group ($P < 0.05$). The remaining CEUS enhancement characteristics, including the proportions of enhancement at arterial phase, enhancement at late phase, tumor necrosis, and capsule enhancement, were similar between the two groups. The differences were not statistically significant ($P > 0.05$; Table 6).

DISCUSSION

Imaging examination plays an important role in tumor discovery, auxiliary diagnosis, treatment, and follow-up. B-mode ultrasound and CEUS are widely used in clinical practice as non-invasive and simple imaging methods. However, due to the rareness of hNEN, there is currently little experience in imaging diagnosis of hNEN, which may result in clinicians not being able to obtain correct imaging results for hNEN, thus affecting the diagnosis and treatment of hNEN. Therefore, the present study first compared the B-mode ultrasound and CEUS performance between hNEN and HCC. Then, we compared the B-mode ultrasound and CEUS characteristics of different sources of hNEN and different malignant degrees of hNEN in order to report clinical diagnostic experience for hNEN.

Comparison of B-mode ultrasound and CEUS results between hNEN and HCC groups

Recent studies have reported that the characteristics of hNEN B-mode ultrasound are uniform hyperechoic or hypoechoic masses with clear boundaries[12,13]. Most of hNEN CEUS characteristics are "fast forward and fast out"[14]. Centripetal enhancement at the arterial phase appears first, and then uniform high enhancement appears[15]. The characteristics of HCC B-mode ultrasound are hypoechoic or mixed echo masses with clear boundaries[16]. The CEUS characteristics are “fast forward and fast out” as well. But most of the CEUS characteristics of HCC showed uniform high enhancement at the arterial phase[17-19]. In this study, there was no significant difference in lesion size and the proportions of different lesion property and posterior echo attenuation in the comparison of B-mode ultrasound and CEUS results between hNEN and HCC

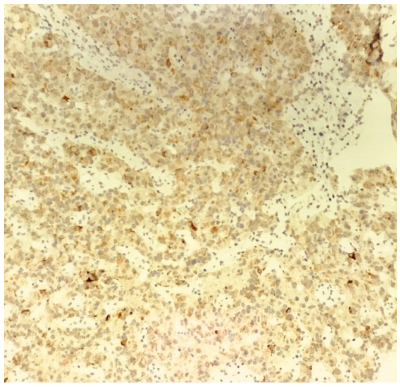

Figure 2 ChrA positive expression in hepatic neuroendocrine neoplasm.

groups. The possible reason is that both hNEN and HCC are solid and blood-rich tumors[20]. They have similar characteristics in B-ultrasound signs and enhancement features. However, the proportions of multiple liver lesion, unclear border, and high echo lesion in the hNEN group were higher than those in the HCC group. The proportions of non-uniform echo and peripheral acoustic halo in the hNEN group were lower than those in the HCC group. It has been suggested that if the liver lesions found in the ultrasound examination are multiple, uniform high echo, and without peripheral acoustic halo, it may be hNEN. Further examination should be performed to determine if there are extrahepatic lesions.

In the comparison of CEUS results, the initial enhancement time was similar between the hNEN and HCC groups, but the washout to iso-enhancement time and washout to hypo-enhancement time in the hNEN group were lower than those in the HCC group. These findings indicated that the washout time in hNEN was earlier than that in HCC. The possible reason is that hNENs are transferred from different sources. The blood flow supply composition is different, which results in a different washout time than HCC[21,22]. In addition, the proportions of CEUS characteristics, including enhancement of arterial phase, portal venous phase, and enhancement of late phase, enhancement forms, tumor vasculature, tumor necrosis, and capsule enhancement, were similar in the hNEN and HCC groups. Because the CEUS enhancement features of hNEN and HCC are similar, it is difficult to distinguish clinically. It is necessary to pay special attention to the difference of contrast agent washout time between hNEN and HCC.

Therefore, this study suggests two points in the ultrasound examination: (1) Intrahepatic lesions are multiple, uniform, and high echo and without peripheral acoustic halo; and (2) In the CEUS performance of intrahepatic lesions, the uniform high enhancement at arterial phase was found, and the washout is rapid. The diagnosis of hNEN needs to be considered.

Comparison of B-mode ultrasound and CEUS results among hNENs from different sources

hNEN can be transferred from multiple sites, including the pancreas, gastrointestinal tract, liver, lungs, adrenal glands, *etc*. Gastroenteropancreatic NENs are the main source of hNEN[23]. Previous studies have revealed that although the treatment of hNEN is surgery, the efficacy and 5-year survival of different sources of hNEN are different[24-26]. The survival time of hNEN patients from the gastrointestinal tract is significantly longer than that of hNEN patients from the pancreas[27]. Ablation, embolism, and liver transplantation have different effects on hNEN from different sources[28,29]. In addition, some patients with hNEN need to undergo surgery again to remove the primary lesion because they have misjudged the source of hNEN before surgery[30-33]. Therefore, predicting the possible primary site of hNEN is important in guiding the patient's examination, such as finding the extrahepatic primary tumor and the treatment plan. This study analyzed B-mode ultrasound and CEUS results of hNENs from the gastrointestinal tract, pancreas, and other sites. We found there was no significant difference in B-mode ultrasound and CEUS characteristics of hNEN from different sources. All of them were mainly multiple hyperechoic lesions, and the CEUS showed uniformly high enhancement at arterial phase and rapid washout. This indicated that it is difficult to identify hNEN from different sources only by ultrasound. Therefore, this study suggests that when hNEN is suspected to be a metastatic tumor, the pathological examination should be performed to clarify the primary lesion to prevent missed diagnosis.

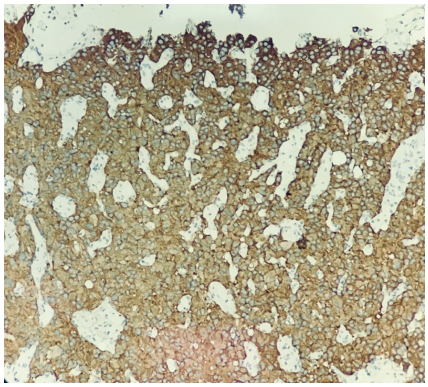

Figure 3 Syno positive expression in hepatic neuroendocrine neoplasm.

Comparison of B-mode ultrasound and CEUS results between hNET and hNEC groups

All hNENs have malignant potential[34-37], and hNEN can be divided into poorly differentiated hNEC (G3 grade) and highly differentiated hNET (G1 and G2 grade) according to its degree of differentiation[38-40]. Most hNET patients require local therapy, and most hNEC patients require systemic therapy[41-43]. Therefore, accurate identification of hNEC and hNET has great significance for clinical treatment of patients. In this study, the characteristics of B-mode ultrasound of hNEC and hNET groups were compared. It was found that both hNEC and hNET groups showed multiple solid lesions, uniform and high echo, no posterior echo attenuation, and peripheral acoustic halo. The difference was not obvious. When comparing CEUS features, it was found that the initial enhancement time, washout to iso-enhancement time, and washout to hypo-enhancement time were similar between the two groups.

The proportions of enhancement at arterial phase, enhancement at late phase, tumor necrosis, and capsule enhancement were similar as well. However, there were differences in the enhancement level at the portal vein phase. It was low enhancement in the hNEC group, while some of the hNETs showed partial equal enhancement. In addition, the proportion of combined tumor vasculature in the hNEC group was larger than that in the hNET group. The possible reason is that hNEC is mainly supplied by arteries, and washout is fast at the portal venous phase. Compared with hNEC, hNET has more portal blood supply, which leads to equal enhancement at the portal venous phase. This is consistent with the biological behavior and malignancy of the tumor[44]. It also explains to some extent why hNEC has a low enhancement level in the portal venous phase and a large proportion of tumor blood vessels[45,46]. In addition, compared with hNET, hNEC has more non-uniform enhancement form at the portal venous phase, probably because hNEC is more prone to cystic lesions, resulting in non-uniform enhancement in CEUS[15,47,48]. Therefore, when the CEUS result of hNEN is equal enhancement at the portal venous phase and uniform enhancement form, hNET can be considered. If there is low enhancement at the portal venous phase, non-uniform enhancement form, and combined tumor vasculature, hNEC should be highly suspected. Further medical treatment measures should be taken.

Limitations and perspectives

Because patients with hNEN are rare, there are currently few targeted studies about hNEN. The number of patients recruited in this study was limited. Patients with primary hNEN were not included in this study. There are further research plans to conduct a multi-center study to collect detailed data from hNEN patients to make the results more comprehensive.

Conclusion

In summary, this study compared the ultrasound characteristics between hNEN and HCC and among hNENs from different sources and malignant degrees. We found that compared with HCC, hNEN showed multiple intrahepatic lesions, uniform high echo, uniform high enhancement at the arterial phase, and rapid washout. The ultrasound characteristics of hNENs from different sources were similar. The low enhancement at portal venous phase, overall non-uniform enhancement form, and the proportion of combined tumor vasculature in hNEC were larger than those of hNET, indicating that hNEC and hNET can be initially identified based on CEUS results.

Table 1 Comparison of B-mode ultrasound characteristics between hepatic neuroendocrine neoplasm and hepatocellular carcinoma groups, *n* (%)

B-mode Ultrasound characteristics		hNEN group, *n* = 55	HCC group, *n* = 55	t/χ^2	*P* value
Diameter in cm		4.32 ± 1.38	3.91 ± 1.27	1.621	0.108
Number of liver lesions	Single	19 (34.5)	47 (85.5)	29.697	0.000
	Multiple	36 (65.5)	8 (14.5)		
Lesion property	Solid	50 (90.9)	47 (85.5)	0.785	0.376
	Cyst	5 (9.1)	8 (14.5)		
Boundary	Clear	29 (52.7)	40 (72.7)	4.705	0.030
	Unclear	26 (47.3)	15 (27.3)		
Echo level	High	28 (50.9)	13 (23.6)	9.498	0.023
	Low	17 (30.9)	24 (43.6)		
	Mixed	10 (18.2)	17 (30.9)		
	Equal	0 (0.0)	1 (1.8)		
Echo uniformity	Uniform	39 (70.9)	26 (47.3)	6.356	0.012
	Non-uniform	16 (29.1)	29 (52.7)		
Posterior echo attenuation	Yes	6 (10.9)	2 (3.6)	-	0.271[1]
	No	49 (89.1)	53 (96.4)		
Peripheral acoustic halo	Yes	13 (23.6)	27 (49.1)	7.700	0.006
	No	42 (76.4)	28 (50.9)		

[1]Represent Fisher's exact test. hNEN: Hepatic neuroendocrine neoplasm; HCC: Hepatocellular carcinoma.

Table 2 Comparison of contrast-enhanced ultrasound characteristics between hepatic neuroendocrine neoplasm group and hepatocellular carcinoma group, *n* (%)

Contrast-enhanced Ultrasound characteristics		hNEN group, *n* = 55	HCC group, *n* = 55	t/χ^2	*P* value
Initial enhancement time in s		16.23 ± 5.29	16.52 ± 5.17	0.291	0.772
Washout to iso-enhancement time in s		26.91 ± 15.39	47.26 ± 16.84	6.615	0.000
Washout to hypo-enhancement time in s		59.84 ± 37.91	99.63 ± 61.82	5.092	0.000
Enhancement level at arterial phase	High	53 (96.4)	55 (100)	2.037	0.154
	Equal	2 (3.6)	0 (0)		
	Low	0 (0)	0 (0)		
Enhancement level at portal venous phase	Equal	7 (12.7)	11 (20.0)	1.063	0.303
	Low	48 (87.3)	44 (80.0)		
Enhancement level at late phase	Equal	2 (3.6)	1 (1.8)	0.343	0.558
	Low	53 (96.4)	54 (98.2)		
Enhancement forms	Fast forward and fast out	53 (96.4)	51 (92.8)	0.705	0.401
	Equal/slow forward and fast out	2 (3.6)	4 (7.2)		
Enhancement forms	Uniform	33 (60)	39 (70.9)	1.447	0.229
	Non-uniform	22 (40)	16 (29.1)		
Tumor vasculature	Yes	34 (61.8)	38 (69.1)	0.643	0.423
	No	21 (38.2)	17 (30.9)		
Tumor necrosis	Yes	16 (29.1)	19 (34.5)	0.377	0.539
	No	39 (70.9)	36 (65.5)		
Capsule enhancement	Yes	7 (12.7)	2 (3.6)	3.025	0.082
	No	48 (87.3)	53 (96.4)		

hNEN: Hepatic neuroendocrine neoplasm; HCC: Hepatocellular carcinoma.

Table 3 Comparison of B-mode ultrasound characteristics transferred from different sources of hepatic neuroendocrine neoplasm, *n* (%)

B-mode Ultrasound characteristics		gastrointestinal tract, *n* = 20	Pancreas, *n* = 29	Other sites, *n* = 6	F/χ^2	*P* value
Diameter in cm		3.24 ± 1.96	2.98 ± 1.95	3.41 ± 2.06	1.772	0.163
Number of liver lesions	Single	7 (35)	10 (34.5)	2 (33.3)	-	1.000[1]
	Multiple	13 (65)	19 (65.5)	4 (66.7)		
Lesion property	Solid	19 (95.0)	25 (86.0)	6 (100.0)	1.781	0.410
	Cyst	1 (5.0)	4 (13.8)	0 (0)		
Boundary	Clear	10 (50.0)	15 (51.7)	4 (66.47)	-	0.856[1]
	Unclear	10 (50.0)	14(48.3)	2 (16.7)		
Echo level	High	6 (30.0)	17 (58.6)	4 (66.7)	-	0.228[1]
	Low	10 (50.0)	7 (24.1)	1 (33.3)		
	Mixed	4 (20.0)	5 (17.2)	1 (33.3)		
	Equal	0 (0)	0 (0)	0 (0)		
Echo uniformity	Uniform	15 (75.0)	20 (69.0)	4 (66.7)	-	0.916[1]
	Non-uniform	5 (25.0)	9 (31.0)	2 (33.3)		
Posterior echo attenuation	Yes	2 (10.0)	4 (13.8)	0 (0)	-	1.000[1]
	No	18 (90.0)	25 (86.2)	6 (100.0)		
Peripheral acoustic halo	Yes	16 (80.0)	21 (72.4)	5 (83.3)	-	0.900[1]
	No	4 (20.0)	8 (27.6)	1 (16.7)		

[1]Represent Fisher's exact test.

Table 4 Comparison of contrast-enhanced ultrasound characteristics transferred from different sources of hepatic neuroendocrine neoplasm, *n* (%)

Contrast-enhanced ultrasound characteristics		Gastrointestinal tract, *n* = 20	Pancreas, *n* = 29	Other sites, *n* = 6	t/χ^2	*P* value
Initial enhancement time in s		16.28 ± 5.82	16.83 ± 6.16	15.22 ± 4.92	1.305	0.372
Washout to iso-enhancement time in s		28.82 ± 12.38	27.29 ± 14.92	2 1.83 ± 11.23	0.924	0.477
Washout to hypo-enhancement time in s		64.93 ± 36.29	55.28 ± 31.83	58.21 ± 29.65	0.874	0.592
Enhancement level at arterial phase	High	19 (95.0)	28 (96.6)	6 (100.0)	0.335	0.846
	Equal	1 (5.0)	1 (3.4)	0 (0)		
	Low	0 (0)	0 (0)	0 (0)		
Enhancement level at portal venous phase	Equal	2 (10.0)	5 (17.2)	0 (0)	-	0.610[1]
	Low	18 (90.0)	24 (82.8)	6 (100.0)		
Enhancement level at late phase	Equal	1(5.0)	1(3.4)	0 (0)	-	1.000[1]
	Low	19 (95.0)	28 (96.6)	6 (100.0)		
Enhancement forms	Fast forward and fast out	19 (95.0)	28 (96.6)	6 (100.0)	-	1.000[1]
	Equal/slow forward and fast out	1 (5.0)	1 (3.4)	0 (0)		
Enhancement forms	Uniform	13 (65.0)	16 (55.2)	4 (66.7)	-	0.729[1]
	Non-uniform	7 (35.0)	13 (44.8)	2 (33.3)		
Tumor vasculature	Yes	14 (70.0)	17 (58.6)	3 (50.0)	-	0.667[1]
	No	6 (30.0)	12 (41.4)	3 (50.0)		
Tumor necrosis	Yes	4 (20.0)	10 (34.5)	2 (33.3)	-	0.569[1]
	No	16 (80.0)	19 (65.5)	4 (66.7)		
Capsule enhancement	Yes	2 (10.0)	4 (13.8)	1 (16.7)	-	1.000[1]
	No	18 (90.0)	25 (86.2)	5 (83.3)		

[1]Represent Fisher's exact test.

Table 5 Comparison of B-mode ultrasound characteristics between hepatic neuroendocrine tumor and hepatic neuroendocrine carcinoma groups, *n* (%)

B-mode Ultrasound characteristics		hNET group, *n* = 35	hNEC group, *n* = 20	t/χ^2	*P* value
Diameter in cm		4.58 ± 2.91	5.08 ± 3.87	0.543	0.590
Number of liver lesions	Single	12 (34.3)	7 (35.0)	0.003	0.957
	Multiple	23 (65.7)	13 (65.0)		
Lesion property	Solid	31 (88.6)	19 (95.0)	0.636	0.425
	Cyst	4 (11.4)	1 (5.0)		
Boundary	Clear	18 (51.4)	11 (55.0)	0.065	0.799
	Unclear	17 (48.6)	9 (45.0)		
Echo level	High	19 (54.3)	9 (45.0)	0.443	0.801
	Low	10 (28.6)	7 (35.0)		
	Mixed	6 (17.1)	4 (20.0)		
	Equal	0 (0)	0 (0)		
Echo uniformity	Uniform	25 (71.4)	14 (70.0)	0.013	0.911
	Non-uniform	10 (28.6)	6 (30.0)		
Posterior echo attenuation	Yes	5 (14.3)	1 (5.0)	-	0.399[1]
	No	30 (85.7)	19 (95.0)		
Peripheral acoustic halo	Yes	9 (25.7)	4 (20.0)	-	0.749[1]
	No	26 (74.3)	16 (80.0)		

[1]Represent Fisher's exact test. hNET: Hepatic neuroendocrine tumor; hNEC: Hepatic neuroendocrine carcinoma.

Table 6 Comparison of contrast-enhanced ultrasound characteristics between hepatic neuroendocrine tumor and hepatic neuroendocrine carcinoma groups, *n* (%)

Contrast-enhanced Ultrasound characteristics		hNET group, *n* = 35	hNEC group, *n* = 20	t/χ^2	*P* value
Initial enhancement time in s		16.83 ± 5.08	16.28 ± 4.93	0.834	0.854
Washout to iso-enhancement time in s		30.84 ± 10.38	27.68 ± 9.74	1.856	0.804
Washout to hypo-enhancement time in s		65.28 ± 37.84	51.72 ± 31.85	1.152	0.833
Enhancement level at arterial phase	High	33 (94.3)	20 (100.0)	1.186	0.276
	Equal	2 (5.7)	0 (0)		
	Low	0 (0)	0 (0)		
Enhancement level at portal venous phase	Equal	7 (20.0)	0 (0)	4.583	0.032
	Low	28 (80.0)	20 (100.0)		
Enhancement level at late phase	Equal	1 (2.9)	1 (5.0)	-	1.000[1]
	Low	34 (97.1)	19 (95.0)		
Enhancement forms	Fast forward and fast out	34 (97.1)	19 (95.0)	-	1.000[1]
	Equal/slow forward and fast out	1 (2.9)	1 (5.0)		
Enhancement forms	Uniform	28 (80.0)	5 (25.0)	16.042	0.000
	Non-uniform	7 (20.0)	15 (75.0)		
Tumor vasculature	Yes	15 (42.9)	19 (95.0)	14.661	0.000
	No	20 (57.1)	1 (5.0)		
Tumor necrosis	Yes	10 (28.6)	6 (30.0)	0.013	0.911
	No	25 (71.4)	14 (70.0)		
Capsule enhancement	Yes	6 (17.1)	1 (5.0)	-	0.402[1]
	No	29 (82.9)	19 (95.0)		

[1]Represent Fisher's exact test. hNET: Hepatic neuroendocrine tumor; hNEC: Hepatic neuroendocrine carcinoma.

ARTICLE HIGHLIGHTS

Research background

Hepatic neuroendocrine neoplasm (hNEN) is a rare tumor clinically. It is important to identify

the source and malignant degree of hNEN and distinguish it from hepatocellular carcinoma (HCC). Imaging examination is required for the initial screening of hNEN. However, there is a lack of data regarding imaging diagnosis of hNEN.

Research motivation

Because of the lack of imaging examination experience, the screening and identification of hNEN is difficult. Research has revealed that there are some differences among hNEN with different sources and malignant degrees screened by ultrasound and contrast-enhanced ultrasound (CEUS). By analyzing the characteristics of ultrasound and CEUS, our study hopes to provide more helpful information in the diagnosis of hNEN.

Research objectives

In this study, the ultrasound performance between hNEN and HCC and data of hNEN with different sources and malignant degrees were compared. The purpose of this study was to improve the accuracy of the identification of hNEN and provide useful information for its clinical diagnosis.

Research methods

A total of 55 patients with hNEN were recruited, the hNEN group. There were 35 cases in the hepatic neuroendocrine tumor (hNET) group, and 20 cases in the neuroendocrine carcinoma (hNEC) group. About 55 patients with HCC were recruited as the HCC group. The characteristic differences of B-mode ultrasound and CEUS between hNEN and HCC, hNEN from different sources, and between hNEC and hNET were compared and analyzed.

Research results

Compared with the HCC group, the proportions of multiple liver lesions, unclear borders, and high echo lesions were higher and the proportions of non-uniform echo and peripheral acoustic halo were lower in the hNEN group. In the NEN group, the washout to iso-enhancement time and washout to hypo-enhancement time were lower than those of the HCC group. The proportion of low enhancement of portal venous phase, non-uniform enhancement forms, and combined tumor vasculature in the hNEC group was greater than that in the hNEN group.

Research conclusions

Compared with HCC, the ultrasound performance of hNEN showed more intrahepatic lesions, uniform high echo, uniform high enhancement at arterial phase, and rapid washout. Compared with hNET, the CEUS characteristics of hNEC are low enhancement of portal venous phase, non-uniform enhancement forms, and combined tumor vasculature.

Research perspectives

To expand this research, future studies should include more hospitals in order to collect detailed data from more hNEN patients. The ultrasound results of primary hNEN also need to be analyzed further to provide stronger evidence for clinical diagnosis.

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