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EDITORIAL

Multidisciplinary management of gastric and gastroesophageal cancers

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Abstract

Carcinomas of the stomach and gastroesophageal junction are among the five top leading cancer types worldwide. In spite of radical surgical R0 resections being the basis of cure of gastric cancer, surgery alone provides long-term survival in only 30% of patients with advanced International Union Against Cancer (UICC) stages in Western countries because of the high risk of recurrence and metachronous metastases. However, recent large phase-Ⅲ studies improved the diagnostic and therapeutic options in gastric cancers, indicating a more multidisciplinary management of the disease. Multimodal strategies combining different neoadjuvant and/or adjuvant protocols have clearly improved the gastric cancer prognosis when combined with surgery with curative intention. In particular, the perioperative (neoadjuvant, adjuvant) chemotherapy is now a well-established new standard of care for advanced tumors. Adjuvant therapy alone should be carefully discussed after surgical resection, mainly in individual patients with large lymph node positive tumors when neoadjuvant therapy could not be done. The palliative treatment options have also been remarkably improved with new chemotherapeutic agents and will further be enhanced with targeted therapies such as different monoclonal antibodies. This article reviews the most relevant literature on the multidisciplinary management of gastric and gastroesophageal cancer, and discusses future strategies to improve locoregional failures.

Key words: Gastric cancer; Chemotherapy; Chemoradiation; Adjuvant; Neoadjuvant

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INTRODUCTION

Gastric and esophageal cancers are among the leading causes of cancer-related death worldwide. Even if the incidence of distal gastric cancer has been decreasing over the past decades, the incidence of newly diagnosed proximal cancers (localized at cardia and gastro-esophageal junction), including Barrett's carcinoma, has dramatically increased[1]. Despite considerable progress in surgical resection as the primary curative treatment for gastric cancer in Japanese and Western countries, more than half of all patients with advanced UICC stage disease undergoing radical primary tumour resection relapse and die within five years[2]. The prognosis of these curatively resected cancer patients remains poor due to high rates of local recurrences as well as early lymph node and systemic metastases. Therefore, new perioperative, neoadjuvant, adjuvant and palliative chemotherapy strategies are of great importance in handling these patients.

MULTISCIPLINARY STRATEGIES FOR DIAGNOSIS AND STAGING

Until recently, the standard diagnostic approach after endoscopic and histological diagnosis of localized advanced gastric adenocarcinoma was to perform only limited staging procedures with sonography and chest X-ray, followed nearly always by attempted surgical

resection. These limited diagnostic tools resulted in a non-optimal description of the local tumor extension and the detection of regional and distant metastases, often not allowing an optimal treatment selection.

It became accepted during the last decade that the lack of co-operation between different medical disciplines prevented an improvement in available therapies. Patient care often consisted of fragmented strategies and lacked long-term planning. The multidisciplinary management of gastric and gastroesophageal cancers, in diagnosis as well as in treatment strategies, gains now even more ground after results of recent randomised studies became available. The time has come for the launch of multimodal treatments to increase the chance of better outcome, longer survival or even cure[3]. By using this team approach, all diagnostic and therapeutic disciplines, such as the gastroenterologist, surgeon, oncologist, radiologist and radiotherapist, will be instrumental in planning the effective administration of their treatment modalities. The diagnostic arsenal, i.e. CT scan, endoscopic ultrasound (EUS), mini-laparoscopy, MRI and PET, allows improved pre- or postoperative staging[4,5]. For endoscopically large tumors and tumors of the gastro-esophageal junction in particular, CT scan of the abdomen/thorax and EUS are mandatory for an exact preoperative tumor and node metastases staging. EUS allows the differentiation between small and large tumours as well as staging or biopsies of mediastinal and celiac lymph nodes[6,7]. In addition, mini-laparoscopy is a valuable tool, as peritoneal carcinosis is found in about 20% to 30% of all gastric cancer patients at first diagnosis[8]. As PET scan has also been shown to effectively predict clinical response in esophageal and gastric cancer, it might potentially allow better allocations and adjustments for further individualized and optimized treatment strategies[9]. Staging with PET may best be used either in patients with locally advanced disease who may benefit from curative resection, if distant metastases are not found, or in patients with high grade stenosis, where EUS is not applicable.

CURATIVE INTENT-THE OPTIMAL RESECTION

To date, the mainstay of curative treatment of gastric cancer has been radical surgical dissection (ESMO clinical recommendations 2007). However, high rates of local recurrences, early lymph node and systemic metastases highlight the need of further efforts to standardize and optimize the surgical treatment. Thus, the type of resection (subtotal *vs* total gastrectomy) and the role of extensive lymphadenectomy have been subjects of international debates. For distal gastric cancers, subtotal gastrectomy has been shown to have an equivalent oncologic result with significantly fewer complications when compared with total gastrectomy[10]. Even if the surgical procedure of choice for proximal gastric cancers is more controversial, because both proximal gastrectomy and total gastrectomy are associated with postoperative nutritional impairments, the oncologic outcome of patients with proximal gastric cancer is independent of the type of gastric resection performed[11]. Currently, total gastrectomy for proximal (cardia) tumors is recommended in Europe.

The extent of regional lymphadenectomy required for optimal results is still debated. Several prospective randomised trials examining the role of more extended lymph node dissections (D1 *vs* D2) did not find clinically relevant improvements in overall survival. However, the interest in extended lymphatic dissections (D2 and greater) has not waned. A retrospective multicentre observation study in Germany found a significant survival advantage in patients undergoing extended lymphadenectomy[1]. In contrast, however, at least two prospective European trials compared D1 with D2 dissection: one in the Netherlands, by the Dutch Gastric Cancer Group (DGCG), and one in the UK, by the Medical Research Council (MRC)[12,13]. Even though the results have been debated differently, both trials found that extended lymphadenectomy associated with significantly higher morbidity and mortality rates compared with limited lymphadenectomy. Likewise, splenectomy and pancreatectomy were associated with a significantly increased risk of operative mortality. Interestingly, no significant survival difference was found for either group in the final results of the DGCG study after 11 years of follow-up[14]. As defined in this study, for patients with N2 disease an extended lymph node dissection may offer cure, but it remains difficult to identify patients who have N2 disease. Morbidity and mortality are greatly influenced by the extent of lymph node dissection, pancreatectomy, splenectomy and age. Extended lymph node dissections may thus be of benefit if morbidity and mortality can be avoided. Recently, the Japan Clinical Oncology Group (JCOG) presented an ambitious trial comparing D2 lymph node dissection with more extensive lymphadenectomy[15]. Here, the mortality rate was remarkably low (1%). Thus, a surgical option that may decrease postoperative morbidity and mortality is an "over –D1" lymphadenectomy with preservation of the pancreatic tail without splenectomy[16].

With improvements in endoscopic techniques (endoscopic mucosal resection) and minimal access surgery, there has been interest in applying these modalities to early gastric cancer. Node-negative T1 tumors are associated with a 5-year survival of more than 90%[17]. As such, there is interest in performing more limited resection for these tumors. Endoscopic resection should be accepted as the treatment of choice in most patients with high-grade intraepithelial neoplasia and mucosal carcinoma in the esophagus. Low morbidity (1%-3%) and mortality (0%) and better quality of life, due to organ preservation, are points in favor of endoscopic resection and against surgical in cases of early oesophageal carcinoma (Barrett's)[18]. Here proper patient selection is paramount. The probability of lymph node metastasis in early gastric cancer is influenced by tumor factors and is correlated with increasing tumor size, submucosal, lymphatic and vascular invasion and poorly differentiated tumors[19].

To improve the acceptance of endoscopic treatment, further prospective trials with long-term data are necessary. Regardless of the technique used for resecting early gastric cancer, complete excision with negative margins is required.

ADJUVANT STRATEGIES

Because of the high rates of local recurrences and distant metastases, different adjuvant chemotherapy protocols have been compared with surgery alone in advanced gastric cancer in Europe, Asia and the United States. In a recent review of these studies the 5-year survival results suggested only a moderate improvement following adjuvant treatment[20]. However, the majority of the chemotherapeutic regimens used in these studies are regarded as suboptimal today.

Two recent phase Ⅲ trials again support adjuvant chemotherapy. Sasako *et al* examined the adjuvant efficacy of single-agent S-1 compared with surgery alone in a study with 1059 patients with Stage Ⅱ/Ⅲ disease, after potentially curative D2 gastrectomy (ACTS-GC study). After 3-year follow-up and rare grade 3/4 toxicities, overall survival and relapse-free survival favored the S-1 arm, with 81.1% *vs* 70.1% ($P = 0.0015$) and 72.2% *vs* 60.1% ($P = 0.0001$), respectively[21] (Figure 1). The Italian Gruppo Oncologico dell'Italia Meridionale (GOIM) study found also in trend some positive results for epirubicin/etoposide/5-FU/folinic acid (FA) in > D1 resected patients[22].

Adjuvant radiotherapy alone has failed over the last decades to improve treatment results and patient outcome. In the British Stomach Cancer Group trial, no survival advantage has been shown for 436 patients randomized between surgery only and surgery with 45 Gy-50 Gy radiotherapy or surgery with FAM chemotherapy[23]. This debate was further stimulated by the presentation of the SWOG 9008 group study, with combined radiochemotherapy in resected stage IB-IV gastric cancers[24]. After randomisation to either observation or to 2 cycles of FA/5-FU (Mayo-clinic regimen) followed by radiation + FA/5-FU and another 2 cycles FA/5-FU, a statistical significant difference in disease-free and overall survival in favor of the chemoradiation was shown. The absolute increase in median survival of 9 mo was hampered by suboptimal surgery (less than D1 in majority of patients) and radiotherapy; 35% protocol deviations. Additionally, in the multimodality arm, the local relapse rate was reduced from 90% to 29%. However, there was no difference in the risk of distant metastasis for either group. Despite positive data, several concerns have been raised concerning that in both arms the patients had high risk for relapse (more than 2/3 had T3 or T4 tumors and 85% positive lymph node metastases), the suboptimal surgery (54% of below D1) was counterbalanced by adjuvant chemoradiation and the number of patients (only 64%) who received the full schedule of chemotherapy and radiation.

In addition, Park and colleagues investigated a similar protocol in 290 patients, all of whom were curatively resected with extensive D2 lymph node dissection[25]. After a median follow up of 49 mo, 43% of patients relapsed, with 67% local relapses and 36% distant metastases. The five-year overall and relapse free survival rates were 60% and 57%, better than in the SWOG trail, respectively[25]. Therefore, it is still questionable whether Japanese or European patients undergoing D2 resection may benefit of postoperative chemoradiation.

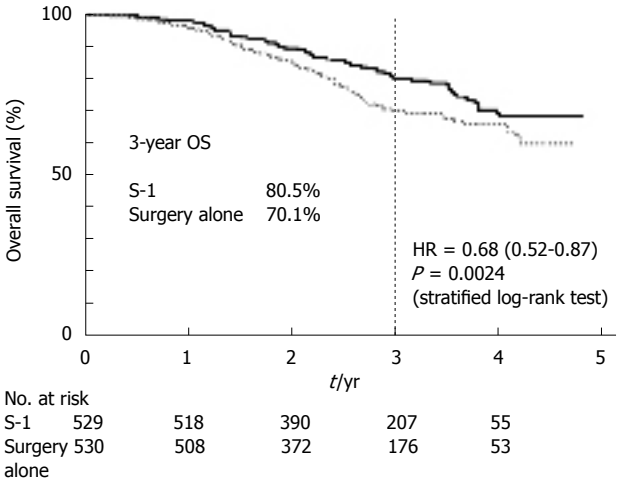

Figure 1 Survival of S-1 monotherapy *versus* surgery alone for stage Ⅱ/Ⅲ gastric cancer patients after curative D2 gastrectomy (ACTS-GC study)[22].

PERIOPERATIVE MULTISCIPLINARY APPROACHES

Neoadjuvant chemotherapy

Many reasonable rationales justify the application of neoadjuvant chemotherapy, which is particularly interesting as a short-term therapy (i.e. two cycles of 6-8 wk) given simultaneously and/or sequentially with radio-chemotherapy. Possible advantages of neoadjuvant therapy +/- adjuvant strategies are: (1) Tumour vascularisation results in higher therapeutic efficacy and downstaging. (2) Excision of chemoradiated areas can result in lower long-term toxicity. (3) Early systemic therapy allows better control of tumour micrometastases. (4) Operation may not be compromised with higher morbidity and mortality.

Neoadjuvant chemotherapy aims at downstaging patients, improving curative resectability of locally advanced disease, and eventually increasing patient survival. It can also provide important information for the postoperative use of chemotherapeutic agents, by evaluating the response of the resected primary tumor, and it is also considered effective in reducing occult micrometastases. Theoretically, introducing chemotherapy at an early phase of the disease may facilitate delivery of drugs towards the primary lesion without impairing vascularization. In addition, major surgery such as total gastrectomy delays the start of postoperative systemic chemotherapy by a month or more, potentially giving microscopic residual diseases an opportunity to proliferate. On the other hand, it has been suggested that patho-

Table 1 Ongoing important phase Ⅲ clinical trials, including monoclonal antibodies and signal transduction/tyrosine kinase inhibitors

Name	Design	Indication
TOGA	XP or FP +/- trastuzumab	Advanced gastric cancer HER2-positive
AVAGAST	XP +/- bevacizumab	Metastatic gastric cancer
REAL-3	EOX +/- panitumumab	Advanced esophagogastric cancer
FFCD 03-07	ECX followed by FOLFIRI *vs* FOLFIRI followed by ECX	Advanced esophagogastric cancer
EXPAND	XP +/- Cetuximab	Advanced/Metastatic gastric cancer
MAGIC-B	Perioperative ECX +/- bevacizumab	Neo-adjuvant gastric cancer
CLASSIC	XELOX *vs* observation	Adjuvant gastric cancer

logical non-staging of the tumor could be the major disadvantage of neoadjuvant strategies. However, since modern imaging technologies such as CT, MRI and EUS plus fine-needle biopsies allow preoperative clinical staging for locoregional lymph node spread, overtreatment of patients with gastric cancer is less likely compared with earlier trials.

Patients responding to neoadjuvant treatment presented with a better performance status during their remission without compromising the subsequent operation with higher morbidity and mortality[26]. Although a number of randomised (mainly phase Ⅱ) studies for neoadjuvant chemotherapy alone have suggested improved survival compared with historical controls, evidence from a randomised phase Ⅲ trial were still missing[27,28].

Apart from the newly updated version of the neoadjuvant MRC trial[29,30], two large phase Ⅲ studies have now clearly proved the preoperative concept to be beneficial for patients with gastric and gastro-oesophageal cancers[31,32]. The recently published MAGIC trial was the first large randomised study of perioperative chemotherapy to be conducted with an adequate follow-up period. It was initiated to compare surgery alone *versus* surgery with perioperative chemotherapy in which patients received three preoperative and three postoperative cycles of ECF[31]. After enrolment of 503 patients with resectable gastric (74%) or lower oesophageal cancer (26%), the proportion of patients with curative resection was larger in the chemotherapy plus surgery arm (79% *vs* 69%, $P = 0.018$). After 5 years, the overall survival rate clearly favored the chemotherapy plus surgery arm over the surgery alone arm (hazard ratio for death, 0.75; 95% CI, 0.60-0.93; $P = 0.009$; 5-year survival rate, 36% *vs* 23%), as did the progression-free survival rate (hazard ratio for progression, 0.66; 95% CI, 0.53-0.81; $P < 0.001$).

The French FFCD (Federation Française de Cancerologie Digestive) Group trial confirmed these important data with their phase Ⅲ study in which they randomized 224 patients to perioperative FUP (5-FU/cisplatin; surgery; 5-FU/cisplatin) or surgery alone. With the same postoperative mortality rates for both arms, the perioperative group presented with significantly higher R0 resection rates. In addition, 3-year disease-free survival increased by 15% (40% *vs* 25%) and 5-year survival improved (38% *vs* 24%)[32].

To generate additional neoadjuvant data on tumors of the gastro-oesophageal junction, three randomised studies for oesophageal cancer included high percentages of adenocarcinomas of the lower oesophagus or the cardia region[33]. In contrast to one large negative phase Ⅲ trial with chemotherapy/surgery *vs* surgery alone[34], one study showed a significant survival benefit and one study found a trend for improved 3-year survival for radiochemotherapy[35,36]. Additionally, the recently updated MRC trial with 802 patients demonstrated a long lasting benefit in median survival (16.8 mo *vs* 13.3 mo) and the increase in 2-year survival of 9% for the chemotherapy group, with no difference in the rate of perioperative death or postoperative complications[29,30].

The identification of an effective chemotherapy regimen and optimal treatment schedule for locally advanced disease has been another important issue in neoadjuvant treatment for gastric cancer. There is optimism that the use of multiple targeted therapies in gastric cancer will produce further improved results. Thus, various phase Ⅰ/Ⅱ clinical trials, including monoclonal antibodies and signal transduction/tyrosine kinase inhibitors for EGFR, monoclonal antibodies to the HER-2/*neu* receptor and VEGF-ligand, and other novel drugs acting on intracellular signaling pathways, are under way, like bevacizumab[37] or cetuximab or panitumumab (Table 1).

Neoadjuvant radiation

With regard to optimizing locoregional tumour control, radiotherapy in the neoadjuvant setting recently came into focus. Preoperative radio-chemotherapy has the advantage that the location of the primary cancer is known more precisely, which facilitates the planning of more accurate and effective radiation fields. In addition, the preoperative approach may allow significant time to observe high-risk patients for future growth of advanced cancers or metastases.

The German Oesophageal Cancer Study Group recently analysed the additional contribution of preoperative radiotherapy to neoadjuvant chemotherapy (POET study)[38] (Figure 2). Patients with locally advanced oesophagogastric adenocarcinomas (Stage T3-T4 NX M0 according to EUS, CT and laparoscopy) were randomised to 2.5 courses of chemotherapy (cisplatin/FA/5-FU weekly) *versus* two courses of the same chemotherapy followed by 3 wk of chemoradiotherapy (30 Gy/cisplatin/etoposide). Despite some increased postoperative mortality after chemoradiotherapy (five *vs* two patients), the median survival (32.8 mo) and the 3-year survival rate (43%) were

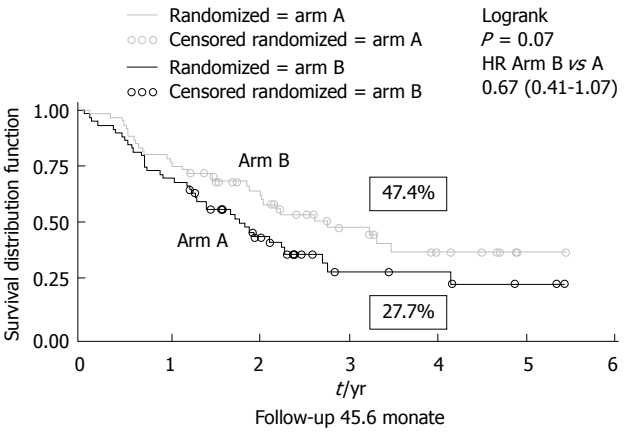

Figure 2 Overall survival of patients with locally advanced oesophagogastric cancers with preoperative neoadjuvant chemoradiation (Arm B) *vs* neoadjuvant chemotherapy alone (Arm A, POET study)[38].

significantly improved in this group compared with patients who received chemotherapy alone (21.1 mo and 27%, respectively).

Pathological responses of oesophageal cancers strongly correlated with disease-free survival after preoperative radio-chemotherapy[39,40]. Ajani *et al* investigated the effects of induction chemotherapy combined with preoperative radio-chemotherapy. Taxanes, cisplatin and 5-FU were followed by radiation with 45 Gy (25 fractions in 5 wk) plus 5-FU infusions[41]. Interestingly, pCR/pPR response rates were 64% in all operated patients, with a significantly longer median survival (64 mo *vs* 30 mo) in patients with pathological remissions. In a second multicentre study of Ajani and colleagues, the pCR and R0 resection rates were 26% and 77%, respectively. At 1 year, more patients with pCR (82%) were alive compared with those with < pCR (69%). Again, these parameters were closely associated with better progression-free and overall survival[42]. Furthermore, it has still to be determined whether any radiation escalation by hyperfractionation (more than one fraction of radiotherapy per day) or acceleration (shortening of treatment duration) may improve local control whilst maintaining a similar risk of late normal tissue damage[43].

In addition to cisplatin/5-FU, new anticancer drugs such as taxanes, irinotecan and oxaliplatin have been reported to induce even higher objective response rates of up to 70% in recent years, and an improvement in overall median survival of up to 12 mo in palliative treatment[44]. The taxanes, docetaxel or paclitaxel promote microtubule stabilisation by increasing tubulin polymerization. As a result, they may also enhance radiosensitivity by causing cell cycle arrest in the G2/M phase[45]. These new chemotherapy regimens may be additional combinations to intensify localized multidisciplinary approaches in resectable or unresectable advanced diseases to further decrease incomplete resection rates as well as morbidity and mortality rates.

Additionally, results of other tumor entities, such as the JCOG 9907 study for esophageal squamous cell cancers, clearly favour a combined modality approach in the neoadjuvant setting[46]. Even more, some randomized trials of chemo-radiotherapy *vs* radiotherapy alone in head and neck, oesophageal or anal cancer showed better locoregional control and overall survival rates for the multimodal protocols. Thus, direct comparisons between neoadjuvant and postoperative adjuvant strategies will be worthwhile in the near future.

TREATMENT OF METASTATIC DISEASE (STAGE IV)

Chemotherapy has increasingly justified its role in the treatment of metastatic disease, with the survival of treated patients being significantly better than that for patients receiving best supportive care. To date, 5-FU derivatives combined with cisplatin have been accepted as the most useful form of palliative chemotherapy, often additionally modulated by combinations with other anticancer drugs, such as epirubicin or leucovorin (FA)[44,47]. In a Cochrane review of randomised trials in advanced gastric cancer, the best survival rates were achieved with anthracyclines, cisplatin and 5-FU, both independently and in combination[48]. Within these combinations, ECF proved to be the best tolerated. Other trials have shown improved overall survival with palliative regimens, such as docetaxel/cisplatin/5-FU[49], oxaliplatin/FA/5-FU[50] and irinotecan/FA/5-FU[51,52]. However, continuous infusion of 5-FU is considered cumbersome because it requires the implantation of central venous catheter and the use of portable infusion pumps, which are associated with complications such as thromboses and wound infections. Capecitabine and S1, prodrugs and oral analogues of 5-FU, can mimic 5-FU continuous infusions and are at least equally effective in tumor control and less toxic than intravenous 5-FU in gastric cancer patients[53,54]. Remarkably, just recently Cunningham and colleagues evaluated capecitabine and oxaliplatin as alternatives to infused 5-FU and cisplatin for untreated advanced esophagogastric cancer and depicted at least similar effectiveness for both regimens[55].

Moreover, the use of multiple targeted therapies renewed hope for more effective and better tolerated chemotherapy regimes in the palliative setting to further improve efficacy and survival. Pinto *et al* combined Cetuximab + FOLFIRI (FOLCETUX) in a phase II study and they demonstrated an overall response rate (ORR) of 44.1%, with a median TTP of 8 mo and a median OS time of 16 mo[56]. The major toxicity appeared to be limited to neutropenia (42.1% of grade 3-4), together with the typical side effects associated with cetuximab (skin 21.1%/grade 3-4). Two additional German AIO trials recently support the efficacy of cetuximab, favouring the analysis of standard therapy with or without EGFR inhibitors in advanced cancers[57,58].

Additionally, response rate (65%), time to disease progression (8.3 mo), and overall survival (12.3 mo) were encouraging when bevacizumab was combined with

Irinotecan/Cisplatin in a multicenter phase Ⅱ study[37]. Ongoing studies testing novel agents will further assess the potential improvement in the treatment of patients with metastatic gastric or gastroesophageal junction adenocarcinoma advanced gastric cancer.

CONCLUSION

With respect to the new perioperative and neoadjuvant achievements in improving the treatment options for advanced gastric cancer, multidisciplinary strategies should be integrated into the daily practice of a patient's work-up[54,59]. Clinical co-operative groups of local comprehensive cancer centers and international study groups, such as the JCOG, SWOG, EORTC, MRC, AIO, FFCD and others, have shown that complex preoperative strategies can be implemented. Thus, patients should be included into the aforementioned innovative studies whenever possible. However, if the local clinical setting does not allow participation in such trials, regionally organized treating physicians, e.g. general practitioner, gastroenterologist, surgeon, radiotherapist and oncologist, should meet regularly, ideally weekly, to decide the multimodal therapeutic concepts, integrating pre-operative and post-operative strategies. With all these clinical and scientific efforts, these treatment strategies will definitely continue to further improve the outcome of gastric cancer patients.

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EDITORIAL

Autoantibodies in primary sclerosing cholangitis

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Abstract

The aetiology of primary sclerosing cholangitis (PSC) is not known and controversy exists as to whether PSC should be denominated an autoimmune disease. A large number of autoantibodies have been detected in PSC patients, but the specificity of these antibodies is generally low, and the frequencies vary largely between different studies. The presence of autoantibodies in PSC may be the result of a nonspecific dysregulation of the immune system, but the literature in PSC points to the possible presence of specific antibody targets in the biliary epithelium and in neutrophil granulocytes. The present review aims to give an overview of the studies of autoantibodies in PSC, with a particular emphasis on the prevalence, clinical relevance and possible pathogenetic importance of each individual marker.

Key words: Primary sclerosing cholangitis; Autoantibodies; Autoimmunity; Antibodies against cytoplasmic constituents of neutrophil; Tropomyosin

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INTRODUCTION

Primary sclerosing cholangitis (PSC) is a chronic inflammatory disease of the intra- and extrahepatic biliary tree leading to progressive bile duct strictures and liver cirrhosis[1]. No effective medical treatment is currently available[2] and PSC is a major indication for liver transplantation[3]. The PSC population is heterogeneous, comprising subgroups of regular "large-duct" PSC, patients with "small-duct" affection only[4] and an "overlap-syndrome" between PSC and autoimmune hepatitis (AIH)[5]. Up to 80% of the PSC patients have concurrent inflammatory bowel disease (IBD)[6]. According to standard endoscopic and histological criteria, the IBD is most often classified as ulcerative colitis (UC), but there is also an association with colonic Crohn's disease (CD)[7,8].

The aetiology of PSC is unknown (Figure 1). Immune responses against self antigens in the bile ducts have been proposed to play an important role in the pathogenesis, although controversy exists as to whether PSC should be denominated an autoimmune or merely immune mediated disease[9]. On one side, there are several lines of evidence supporting classification of PSC as an autoimmune disease[10]. This evidence includes (1) association with other autoimmune diseases in the same individual[11] and first degree relatives[12], (2) infiltration of T-lymphocytes in the portal tracts[13] with restriction in T cell receptor V gene usage[14], (3) a statistical association with particular human leukocyte antigen (HLA) haplotypes[15] and (4) the presence of autoantibodies[16]. On the other side, there is no documented effect of immunosuppressants in PSC[2], and in contrast to the female predominance of many diseases regarded as autoimmune, approximately 2/3 of PSC patients are male[17]. These notions suggest that additional pathogenetic factors may exist (e.g. bile acid toxicity[18]), and to what extent and at what disease stage autoimmune mechanisms contribute to the bile duct damage observed in PSC is not known.

In many autoimmune diseases, autoantibodies serve as markers of disease activity, may aid in the diagnosis of patients, and provide important insight into the pathogenesis. In clinical practice, a good

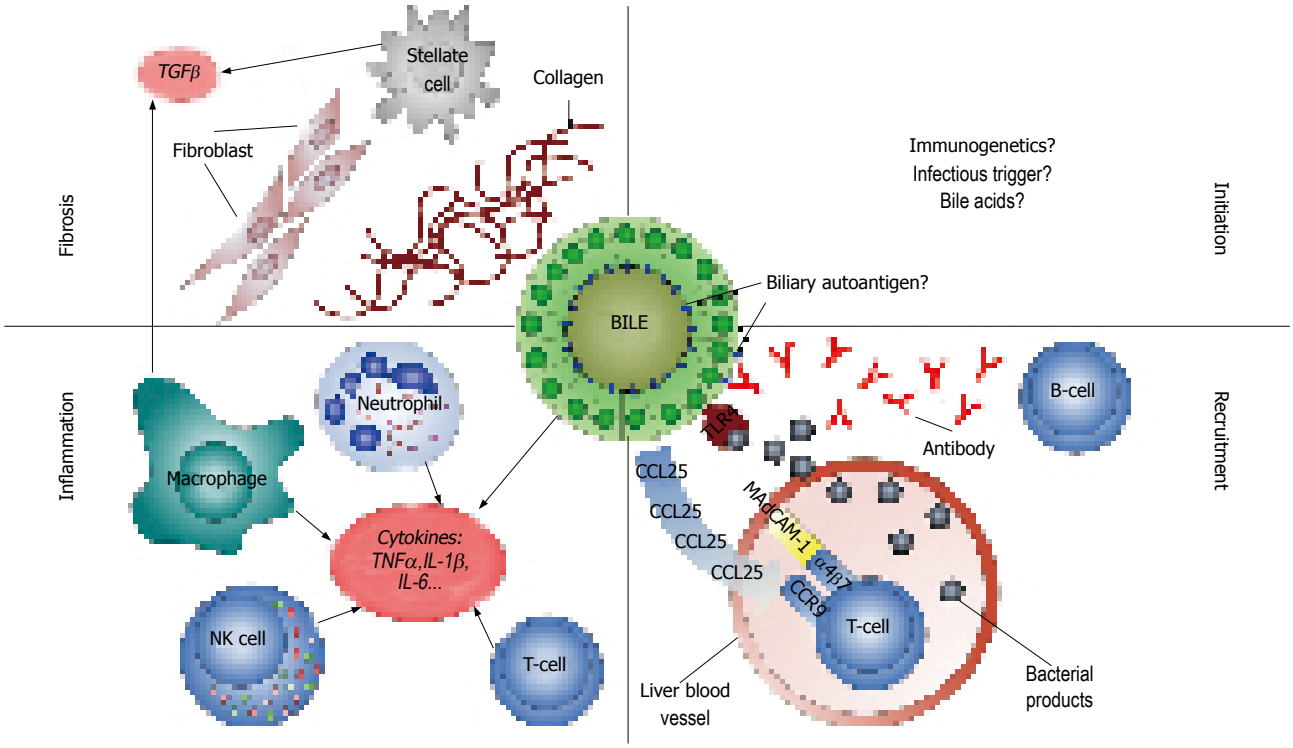

Figure 1 Schematic illustration of key elements of PSC pathogenesis. Initiation (upper right): The initiating factor(s) of PSC pathogenesis are unknown. Immunogenetic factors (including the presentation of autoantigens on PSC associated HLA molecules), an infectious trigger, and toxic or immunological effects from bile acids have been proposed. Recruitment (lower right): Autoantibodies produced by B-lymphocytes bind to biliary epithelial cells (BECs), leading to inflammation when there is concomitant stimulation of toll like receptors (TLRs) by bacterial products [LPS and other pathogen-associated molecular patterns (PAMPs)] from the gut. Recruitment of gut-primed (α4β7) T-lymphocytes in inflammatory bowel disease may contribute to the inflammation because of aberrant expression of the MadCAM-1 ligand in the endothelium and production of the CCL25 chemokine by BECs. Inflammation (lower left): T-lymphocytes and natural killer (NK) cells predominate in PSC affected livers, but neutrophils and macrophages are also recruited. Together with activated BECs they are sources of the cytokines and chemokines that perpetuate the inflammation in PSC. A specific cellular component of neutrophils (tubulin beta 5 chain), has been hypothesized to serve as an autoantigen in this inflammatory process, possibly cross-reacting with the bacterial homolog FtsZ and leading to the generation of anti-neutrophil cytoplasmic antibodies (ANCAs). Fibrosis (upper left): Characteristically in PSC, there is extensive fibrosis and stricturing of the bile ducts. Resulting from the inflammation and possibly concomitant bile leakage, pro-fibrotic factors [e.g. transforming growth factor beta (TGF-β)] from macrophages and/or stellate cells are ultimately responsible for the fibrotic obliteration of the bile ducts and liver cirrhosis in PSC.

marker is sensitive and specific and yields prognostic information [e.g. anti-cyclic citrullinated proteins (anti-CCP) antibodies in rheumatoid arthritis (RA)]. In studies of pathogenetic mechanisms, a good marker is tissue specific and closely linked to other observations regarding the pathogenesis (e.g. TSH receptor antibodies in Graves' disease). In PSC patients, a large number of different autoantibodies have been reported (Table 1). Some of these autoantibodies react with biliary or colonic epithelial antigens, others with constituents of neutrophil granulocytes, and some even with various ubiquitously expressed self antigens.

One of the most consistent findings regarding the aetiology of PSC is the disease association with genetic variants within the HLA-complex on chromosome 6[15]. HLA class I and II genes encode molecules which present antigens to $CD8^+$ and $CD4^+$ T-lymphocytes, respectively, resulting in an immune response against the antigen when appropriate co-stimulation is present[19]. A relationship between particular autoantibodies and disease associated HLA variants has been detected in other autoimmune diseases[20], but in PSC the pathogenetic importance of most of the identified autoantibodies is poorly defined. The present editorial aims to give an overview of the studies of autoantibodies in PSC, with a particular emphasis on the prevalence, clinical relevance and possible pathogenetic importance of each individual marker.

ANTIBODIES AGAINST BILIARY AND COLONIC EPITHELIAL ANTIGENS

The identification of antibodies against well defined biliary antigens in PSC would strongly support the hypothesis of an autoimmune aetiology. Given the high frequency of colitis among the patients, such antigens could potentially also be expressed in the colonic mucosa.

One autoantibody of this type was proposed by Das *et al*[21], who identified an antigenic protein expressed in both colonic and biliary epithelium, in addition to eye, skin and cartilage[22]. This 40 kDa protein was identified as human tropomyosin isoform 5 (hTM5)[23-25]. A monoclonal antibody (Das-1) was developed, and serum from UC and PSC patients inhibited the binding of Das-1 to the epithelium, indicating antibodies

Table 1 Autoantibodies detected in PSC patients

Antibody	Prevalence (%)	(Median)	No. of patients	(Median)	No. of articles
Anti-BEC	63	(63)	30	(30)	1[36]
pANCA	26-94	(68)	13-86	(30)	19 (Table 2)
AMA	0-9	(0)	15-73	(37)	10[44,61,78,89,102,112,137-140]
Anti-LKM	0	(0)	10-80	(37)	7[44,89,112,137,140-142]
Anti-SLA/LP	0	(0)	10-37	(25)	4[44,89,140,142]
ANA[1]	8-77	(30)	13-73	(35)	13[44,61,78,89,99,101,102,111,112,137-140]
SMA[1]	0-83	(17)	10-73	(36)	10[44,61,78,89,111,112,137-140]
ASCA	44	(44)	25	(25)	1[115]
Anti-cardiolipin	4-63	(27)	23-73	(41)	3[61,78,87]
Rheumatoid factor	15	(15)	71	(71)	1[78]
AECA	35	(35)	20	(20)	1[87]
Anti-TPO	16	(16)	73	(73)	1[78]
Anti-GBM	17	(17)	24	(24)	1[87]
Anti-sulfite oxidase	33	(33)	39	(39)	1[130]
Anti-GSTT1	5	(5)	58	(58)	1[133]

PSC: Primary sclerosing cholangitis; Anti-BEC: Antibodies against biliary epithelial cells (measured with flow cytometry); PANCA: Perinuclear antineutrophil cytoplasmic antibodies; ANA: Antinuclear antibodies; SMA: Smooth muscle antibodies; ASCA: Anti saccharomyces cerevisiae antibodies; AMA: Anti-mitochondrial antibodies; Anti-LKM: Liver-kidney microsomal antibodies; Anti-SLA/LP: Antibodies against soluble liver antigen/liver pancreas; AECA: Anti-endothelial cell antibodies; Anti-TPO: Antibodies against thyroid peroxidase; Anti-GBM: Antibodies against the glomerular basement membrane; Anti-GSTT1: Antibodies against glutathione S transferase theta 1. [1]In the largest cohort investigated, ANAs and/or SMAs were detected in 22% (24/111) of the PSC patients, but the frequency of each type was not given[143].

against hTM5 related epitope(-s) in the sera[22,26]. In the cell membrane hTM5 is found complexed with a 200 kDa colonic epithelial protein (CEP), and this complex is speculated to serve as the true target for the Das-1 antibody[27]. Antibodies against hTM5 have been detected in UC patients without PSC[28], and anti-hTM5 in UC sera has recently been shown to induce cytotoxicity against colonic epithelial cells *in vitro*[29]. In PSC patients without concomitant UC, a single study identified antibodies against a 9-amino acid sequence from hTM (not isoform specific) in 100% (8/8) of patients as compared with 69% (33/48) of UC patients and 0/6 PBC patients[30]. The findings of Das *et al* have been partly reproduced by others[31], but given a number of critical concerns[32-35], further studies are required to conclusively confirm and elaborate the importance of the hTM5-CEP antigen in the pathogenesis of PSC.

A Swedish group has reported on the presence of antibodies against isolated biliary epithelial cells (BEC) at high frequencies in sera from PSC (63%) and PBC (37%) patients, *versus* 8% of controls (1/12)[36]. A 40 kDa antigenic protein was identified, but this protein did not react with tropomyosin antibodies, which implies that either the 40 kDa protein in this study is not a tropomyosin isoform or the antibody used reacts with other tropomyosin isoforms. Anti-BEC from PSC sera (and to a lesser extent PBC sera) induced isolated BEC to produce IL-6 and the adhesion molecule CD44, strongly suggesting pathogenetic importance. Recently the group also showed that sera from PSC patients with anti-BEC stimulated BEC to express toll-like receptors (TLR), leading to BEC cytokine production upon exposure to lipopolysaccharide (LPS, endotoxin) from gram negative bacteria[37]. This means that both LPS and antibodies against BEC are necessary to activate BEC and generate cytokine release. An association between the presence of the anti-BEC and PSC associated HLA haplotypes (DR2 and DR3) was also suggested. The relevance of the Swedish findings are further strengthened by a higher frequency of acute liver transplant rejection in patients with anti-BEC prior to transplantation (all liver diseases) than in patients with no anti-BEC[38]. However, it needs to be noted that in this study there was a high prevalence of anti-BEC in all end stage liver patients (HCV 32%, PSC 56%, PBC 75%, HBV 57%, AIH 57%, and alcoholic cirrhosis 71%). This raises concerns as to the PSC specificity of the antibody, which clearly needs to be characterised prior to further studies.

Taken together, the findings of Das *et al* and the Swedish group suggest that antigens expressed in the biliary epithelium may induce self-reactive immune responses under certain conditions. Whether the antigenic epitope(s) lie within the hTM5-CEP complex or elsewhere remains to be elucidated, and the clinical significance of the corresponding autoantibodies must be established.

ANTIBODIES AGAINST NEUTROPHILS

Antibodies against cytoplasmic constituents of neutrophils (ANCAs) were initially described in patients with glomerulonephritis and systemic vasculitis[39,40]. In UC patients, antibodies against nuclear antigens were reported by Calabresi *et al* in 1961[41] and Nielsen *et al* in 1983 (granulocyte specific-ANA)[42]. In PSC such antibodies were reported by Snook *et al* in 1989[43]. These antibodies are also present in a large proportion of patients with AIH[44] and the name ANCA was applied due to the close resemblance to ANCAs found in several of the vasculitides[45,46]. ANCA is analyzed by incubating fixated human neutrophil slides with patient serum, and subsequently with secondary antibodies conjugated to a fluorophore. The indirect immunofluorescence

Table 2 Prevalence of pANCA[1] in PSC patients and controls[2] [% ($n^{\text{antibody positive}}/n^{\text{total population}}$)]

Authors	PSC	PSC -IBD	PSC+IBD	UC -PSC	CD-PSC	AIH	PBC	HC	MT
Terjung *et al*[44]	94[3]					81[4] (142/175)	31	0	IIF
	(33/35)						(14/45)	(0/19)	1:10
Klein *et al*[54]	87			78	27			0	IIF
	(26/30)			(18/23)	(16/60)			(0/20)	1:10
Mulder *et al*[102]	79	77[5]	82[5]			88[6]	28	5	IIF
	(19/24)	(10/13)	(9/11)			(21/24)	(7/25)	(12/252)	1:32
Lo *et al*[56]	77[7]			33	0	33[6]	0	0	AP
	(23/30)			(15/45)	(0/32)	(1/33)	(0/14)	(0/50)	1:10
Seibold *et al*[98]	775	40[8]	88	83	25	33[6]	28	0	IIF
	(17/22)	(2/5)	(15/17)	(38/46)	(20/80)	(5/15)	(7/28)	(0/30)	1:10
Gur *et al*[87]	75	75	75[5]						IIF
	(15/20)	(3/4)	(12/16)						1:20
Muratori *et al*[144]	75					31[4]	2	0	IIF
	(18/24)					(12/39)	(1/51)	(0/18)	1:20
Seibold *et al*[84]	72	50	76[5]	62	4	35[4]	28	0	IIF
	(18/25)	(2/4)	(16/21)	(30/48)	(2/48)	(8/23)	(6/21)	(0/40)	1:10
Zauli *et al*[137]	72								IIF
	(33/46)								-[9]
Hardarson *et al* [145]	69	75	67[5]	76	8	50[6]	0		IIF
	(20/29)	(6/8)	(14/21)	(16/21)	(2/25)	(10/20)	(0/33)		1:40
Roozendaal *et al*[99]	67								IIF
	(46/69)								1:40
Bansi *et al*[146]	66[10]					65[6]	13	0	AP
	(57/86)					(11/17)	(7/55)	(0/36)	1:5
	51[10]					65[6]	11	0	IIF
	(44/86)					(11/17)	(6/55)	(0/36)	1:5
Bansi *et al*[101]	65	29	70[5]	45				0	AP
	(41/63)	(2/7)	(39/56)	(38/85)				(0/36)	1:05
Tervaert *et al*[88]	62					71[6]	33	0	IIF
	(8/13)					(5/7)	(5/15)	(0/24)	-[9]
Roozendal *et al*[57]	49					70[11]	15	0	IIF
	(27/55)					(62/88)	(8/53)	(0/78)	1:40
Claise *et al*[53]	44	25	60	37	15	24[11]	0	0	IIF
	(12/27)	(3/12)	(9/15)	(18/49)	(11/75)	(25/105)	(0/30)	(0/50)	1:20
Vermeulen *et al*[147]	44			56	15	46[6]		5	IIF
	(16/36)			(56/100)	(15/100)	(17/37)		(5/105)	1:40
Wilschanski *et al*[60]	29								IIF
	(7/24)								1:20
Pokorny *et al*[100]	26	29	23			22[6]	0		IIF
	(10/39)	(5/17)	(5/22)			(2/9)	(0/7)		1:20

PSC: Primary sclerosing cholangitis; IBD: Inflammatory bowel disease; UC: Ulcerative colitis; CD: Crohn's disease; AIH: Autoimmune hepatitis; PBC: Primary biliary cirrhosis; HC: Healthy controls; MT: Method and titre considered positive (cut-off); IIF: Indirect immunofluorescence; AP: Alkaline phosphatase method. [1]No distinction between classical/atypical; [2]The single largest study of autoantibodies in PSC reported ANCAs among 84% (61/73) of the patients, but this study did not apply IIF, meaning that this figure is the total of patients with any ANCA subspecificity[78]; [3]Atypical pANCA (as opposed to classical pANCA or cANCA); [4]autoimmune hepatitis type 1 and 2; [5]Calculated from article values; [6]Autoimmune hepatitis not subclassified (1 or 2); [7]ANCA "type 1 pattern" is interpreted as pANCA, including both IgA/IgM/IgG; [8]Significant difference between PSC +IBD and PSC -IBD (*P* value not given); [9]Details not given, correspondence, not peer-reviewed; [10]Calculated sum of 4 patient populations from different countries; [11]Autoimmune hepatitis type 1.

(IIF) pattern is classified as cytoplasmic (cANCA) or perinuclear (pANCA)[47,48]. Billing *et al*[49] and Terjung *et al*[50-52] have made an additional contribution to this nomenclature, documenting that the main ANCA pattern in PSC, AIH and UC is "atypical". This means that the likely antigen is located in the nucleus rather than in the cytoplasm. The names anti-neutrophil nuclear antibodies (ANNAs)[51] and nuclear anti-neutrophil antibodies (NANAs) have thus been proposed[49].

The prevalence of ANCA (subtype not specified) in PSC patients ranges from 42% to 93%[45,53-61], and that of the pANCA subtype from 26% to 94% (Table 2). Comparable prevalences of ANCA are reported in AIH and UC (Table 2). No definite evidence links ANCA to the genetic susceptibility of PSC in terms of particular HLA haplotypes[62]. One study has reported on an increased prevalence of ANCA in PSC relatives as compared with healthy controls[63] while another study could not confirm this[64].

Nuclear specificities of the neutrophil antigens

Multiple neutrophil antigens contribute to different ANCA IIF patterns (Table 3). A study published in abstract form by Terjung *et al*[65] in 2005 proposed that the main antigen of atypical pANCA in AIH, UC and PSC patients is tubulin beta 5 chain (TBB5), a nuclear membrane-associated protein present in myeloid cell lines. Further studies of anti-TBB5 are necessary to characterise the clinical and pathogenetic relevance of these findings. Other nuclear antigens have also been

Table 3 Prevalence of antibodies against a selection of specific neutrophil antigens in PSC patients

Antibody	Frequency range % (median)	No. of patients range (median)	Number of studies
Anti-lactoferrin	4-50 (29)	12-76 (24)	10[55,57,84,85,87,88,99,102,137,144]
Anti-myeloperoxidase	0-33 (2)	12-73 (40)	7[57,78,84,85,87,99,102]
Anti-BPI	5-46 (29)	36-76 (69)	5[55,57, 59,78,99]
Anti-cathepsin G	0-35 (21)	14-76 (55)	5[55,57,84,87,99]
Anti-proteinase 3	0-44 (4)	25-73 (62)	5[57,78,87,99,102]
Anti-elastase	0-35 (9)	23-76 (69)	4[55,87,99,102]
Anti-α-enolase	11-33 (27)	15-55 (36)	3[57,89,147]
Anti-catalase	16-60 (38)	15-55 (35)	2[57,89]
Anti-α-antigen	33 (33)	12 (12)	1[85]
Anti-h-lamp-2	71 (71)	73 (73)	1[78]
Anti-TBB5[1]			

PSC: Primary sclerosing cholangitis; Anti-BPI: Antibodies against bactericidal/permeability increasing protein; Anti-h-lamp-2: Antibodies against human lysosomal-associated membrane protein 2; Anti-TBB5: Antibodies against Tubulin beta-5 chain. [1]No prevalence studies published.

proposed as nuclear targets of pANCA in AIH and UC, notably the high mobility group (HMG) non-histone chromosomal proteins HMG1 and HMG2[66-68] and Histone H1[69]. These have not been studied in patients with PSC.

Cytoplasmic specificities of the neutrophil antigens

A variety of cytoplasmic proteins have also been proposed to be targets for ANCAs in PSC. In ANCA-associated small vessel vasculitis (Wegener's disease, microscopic polyangiitis and Churg-Strauss syndrome) the main proportion of specific ANCAs are directed against proteinase 3 (PR3, mainly cytoplasmic IIF pattern) and myeloperoxidase (MPO, mainly perinuclear IIF pattern)[70]. In these diseases, increased ANCA levels may predict clinical relapse, but there is limited correlation between titres and disease activity. The prevalence of anti-PR3 and anti-MPO in PSC patients is low (Table 3).

Bactericidal/permeability increasing protein (BPI) has functional domains which bind the inner core region of LPS[71]. This binding triggers anti-bacterial activity, neutralization of endotoxin and delivery of endotoxin rich particles to host cells[72]. Anti-BPI is detected in many clinical settings. In PSC, anti-BPI has been found in 5% to 46% of the patients (Table 3), which is similar to UC (3%-39%)[59,73-77], compared with 0% to 5% of healthy controls[57,78]. Anti-BPI is also reported in RA, systemic lupus erythematosus (SLE) and systemic sclerosis[79], and interestingly there is a high prevalence of anti-BPI in cystic fibrosis patients colonized with gram negative bacteria[80].

Another LPS-binding ANCA target is lactoferrin, which is released from neutrophils during inflammation and has bactericidal and immune modulating effects[81]. Antibodies against lactoferrin have been detected in several autoimmune diseases including RA[82], SLE[83], reactive arthritis[82] and ankylosing spondylitis. The reported prevalence of anti-lactoferrin in PSC (4%-54%, Table 3) is similar to that in UC (4%-50%), and considerably higher than in CD (0%-9%)[73,75,76,84-86] and healthy controls (0%)[87,88].

Antibodies against the proteases elastase and cathepsin G are found in up to 35% of patients with PSC (Table 3). Catalase prevents cell damage from reactive oxygen-derived free radicals, and antibodies against catalase have been detected in up to 60% of PSC patients, compared with up to 10% of healthy controls[57,89]. Finally, human lysosomal-associated membrane protein 2 (h-lamp-2) is a target of ANCA in vasculitides[90]. In a single study, anti-h-lamp-2 was detected in a large proportion of PSC patients (71%) *versus* only 15% of healthy controls[78]. No disease controls were investigated. This finding has not yet been reproduced.

Pathogenetic role of ANCAs

The large range of different ANCAs in PSC (Table 3) has been critically interpreted as the ANCAs serving as nonspecific epiphenomena of an immune response against dying neutrophils at an inflammatory site[91,92]. ANCAs (i.e. anti-MPO and anti-PR3) may, however, activate neutrophils[70], and anti-BPI may inhibit clearance of LPS[93]. Also, widely and even ubiquitously expressed antigens sometimes serve as antigens in tissue specific autoimmunity [e.g. anti-mitochondrial antibodies (AMAs) in PBC].

Another possibility is related to the predominant theory on UC and CD, which involves an aberrant response to gut luminal antigens in genetically susceptible hosts[94]. A series of antibodies against bacterial antigens have been detected in IBD patients, and ANCAs may represent such antibodies[94]. One study from 1995 indicated that colonic lamina propria B-cells in UC produce pANCA[95]. In another study, absorption of human pANCA-positive sera with enteric bacterial antigens reduced or abolished the specific perinuclear staining[96]. The targets of these pANCAs are not known, but a study published in abstract form in 2006 indicates that antibodies giving rise to the atypical pANCA pattern have dual reactivity against both TBB5 and the microbial tubulin FtsZ[97]. How these cross-reacting antibodies may lead to hepatobiliary pathology can only be speculated upon.

Diagnostic and clinical relevance of ANCAs

The sensitivity of ANCA in PSC is high in some studies,

whereas specificity is low. In one study of the diagnostic precision of autoantibodies in liver diseases, atypical pANCA with cut-off titre 1:40 had a specificity of 78% and sensitivity of 61% for PSC (AUC, 0.69; 95%CI, 0.61-0.77)[44]. Identification of the principal antigenic target of ANCAs in PSC would allow prospective studies to define this diagnostic role further. Currently, ANCA does not contribute diagnostically or during the clinical follow-up of PSC patients.

In terms of correlation between ANCA and particular clinical characteristics of PSC, no clear interpretation can be made from available data. If ANCAs were to represent markers for intestinal affection in PSC, a higher ANCA prevalence should be detected in PSC patients with IBD than in patients without IBD. This has only been shown in one small study by Seibold *et al*[98]. In another study, anti-lactoferrin was more prevalent in PSC with UC than without[99]. A few papers relate ANCA positivity to biliary tract complications like biliary calculi or cholangiocarcinoma[100], or more extensive involvement of the biliary tree (both intra- and extrahepatic as compared with intrahepatic only)[101]. The presence of pANCA has also been found to correlate with disease stage (cirrhosis or liver transplantation)[100,102], and in one study anti-BPI and anti-cathepsin G were more prevalent in PSC patients with cirrhosis[99]. Most other papers reported no difference in ANCA positivity between early and advanced PSC, and found no correlation between titres and disease activity[45,57,99,103]. ANCAs seem to persist after liver transplantation[98,104], even though the titres may vary during follow-up[103].

AUTOANTIBODIES SPECIFIC TO LIVER DISEASES OTHER THAN PSC

AMA may be considered one of the most useful autoantibodies in the diagnosis of cholestatic liver disease, since AMAs are virtually absent in PSC patients (Table 1) compared with a 90%-95% prevalence in PBC[105]. The AMA antigens are different epitopes of the pyruvate dehydrogenase complex (PDC), especially the PDC-E2[106,107]. Mitochondrial antigens are expressed in all nucleated cells, and AMAs are classically detected by IIF. The presence of AMAs in PBC is an example of how autoimmunity against a ubiquitous antigen may be involved in the pathogenesis of a highly tissue specific disease. One of several proposed theories in PBC hypothesizes that in biliary epithelial cells the main AMA-antigen (PDC-E2) is not glutathiolated (as opposed to in other cells), causing persisting antigenicity of PDC-E2 when biliary epithelial cells undergo apoptosis[108]. Modification of AMA antigens in the liver by xenobiotics may also contribute[108]. A similar post-translational modification of proteins is known to contribute to antigenicity in several autoimmune diseases (e.g. antibodies against citrullinated proteins in RA)[109].

Anti-liver kidney microsomes type 1 (anti-LKM1), anti-soluble liver antigen/liver pancreas antigen (anti-SLA/LP) and anti-liver cytosolic protein type 1 (anti-LC1) are autoantibodies used in diagnosis of AIH[105]. These have not been detected in PSC patients (Table 1).

ANTINUCLEAR (ANAS) AND SMOOTH MUSCLE ANTIBODIES (SMAS)

ANA and SMA are directed against ubiquitous antigens. ANA is the hallmark of SLE and other connective tissue diseases, but are also among the most prevalent autoantibodies in AIH[110]. ANAs may represent a large number of nuclear targets while SMAs are similarly undefined and directed against actin and other cytoplasmic filamentous proteins. ANA is reported in 8%-77% of the PSC patients (Table 1). No particular ANA subspecificities seem to predominate; anti-dsDNA has been reported in 3%-29%[61,78,87,111], anti-ENA in 4%-12%[78,111,112], anti SSA/B in 1%-28%[78,87] and anti-RNP, anti-SCL70, anti-Sm and anti-ssDNA in a minority of patients[87]. SMAs have been reported in 0%-83% of PSC patients (Table 1) but the prevalence is also high in AIH, various malignancies and infections[107]. ANA and SMA often co-exist, they lack organ and disease specificity, and should probably be concluded as irrelevant for the diagnostic process and pathogenesis in PSC.

ANTIBODY AGAINST SACCHAROMYCES CEREVISIAE (ASCA)

ASCA is an antibody against baker's yeast (microbial antigens) and therefore does not represent a typical autoantibody. ASCA was first described in patients with CD in 1988[113]. The antigenic epitope of ASCA is located on the S. cerevisiae mannan, which is a polymer of mannose[114]. In a single study, 44% (11/25) of PSC patients were ASCA positive (57% with concurrent IBD and 39% without IBD) compared with 23% (28/123) of PBC and 18% (12/67) of AIH patients[115]. The presence of ASCA is interesting as a specific example of immune responses towards gut luminal antigens in IBD. As a serological marker in PSC, however, ASCA does not seem to contribute.

ANTI-PHOSPHOLIPID ANTIBODIES

Anti-phospholipid antibodies are directed against phospholipids or phospholipid associated proteins, and are associated with thromboembolic disease. They are commonly detected in connective tissue disorders (e.g. SLE) but also in 1% to 5% of healthy subjects and during infections[116]. Three studies have investigated the presence of anti-cardiolipin antibodies in PSC with the prevalence ranging from 4% to 63% (Table 1). Interestingly, Angulo *et al*[78] found a positive correlation between anti-cardiolipin titres and Mayo risk score and histological disease stage, and there are anecdotal reports of an elevated risk of thrombosis in PSC patients[117]. An increased risk of hepatic artery thrombosis post liver transplantation has also been proposed[118]. Anti-

cardiolipin antibodies have also been reported at low frequencies in UC (16%-26%)[119-121] and CD (16%-27%) patients[120,121].

OTHER AUTOANTIBODIES

In addition to ANA and SMA, several other non-specific autoantibodies are detected in PSC. Rheumatoid factor is detected in connective tissue diseases, infections and lymphoproliferative diseases[122], but is only found in 15% of PSC patients[78]. Anti-endothelial cell antibodies (AECAs) are directed against antigens in endothelial cells and have been reported in 35% of PSC patients in a single small study[87] but are observed in many other clinical conditions including vasculitis, SLE, systemic sclerosis and IBD[123,124]. The clinical and pathogenetic roles of AECAs are not clear[123,124].

A few autoantibodies in PSC are probably related to co-morbidity. In one study, the prevalence of thyroid diseases in PSC patients was 8%[11]. This probably explains the elevated levels of anti-thyroid peroxidase (Table 3) and other thyroid related antibodies in PSC patients[54,78,87]. An association between PSC and celiac disease has been reported[125-127]. Recently the celiac disease related anti-tissue transglutaminase was detected in 7% of PSC patients in a large pre-transplant cohort from the Mayo Clinic (11/155), *versus* 6% (7/112) of PBC and 35% (15/43) of AIH[128] patients. This may in part be explained by shared susceptibility HLA-alleles (DQ2 and DQ8)[129]. Finally, in a single small study, antibodies against the glomerular basement membrane (anti-GBM) were detected in 17% of patients with PSC, while all healthy controls were negative[87]. The significance of this finding is not known.

Antibodies against sulfite oxidase were detected by Preuss *et al*. in 33% (13/39) of PSC patients compared with 5% (5/96) of PBC and 9% (7/77) of AIH patients[130]. Sulfite oxidase is a mitochondrial enzyme previously thought to be the antigen of anti-M4 (an AMA subtype)[131] but this does not seem to be correct[132]. The authors report lower prevalence in PSC patients treated with UDCA but the role of anti-sulfite oxidase antibodies in PSC remains to be established[130].

Glutathione S-transferase theta 1 (GSTT1) was recently investigated as a candidate autoantigen in PSC by Ardesjö *et al* using immunoscreening[133]. This group created a cDNA library based on mRNA from human ductus choledochus[133]. The GSTT1 antigen was identified screening one single PSC patient serum for antibodies against bacteria expressing the cDNA encoded proteins. Upon testing in a larger population of PSC patients (n = 58), antibodies against GSTT1 were only found in three patients, thus concluding GSTT1 as unlikely to serve as an important autoantigen in PSC. Nevertheless, the study points to the possible need for the application of broader screening methods in the search for autoantigens in PSC. The role of autoantibodies and B-cells in other autoimmune diseases has gained renewed interest the last few years[134], not only as pathogenetic factors[135], but also as therapeutic targets (e.g. Rituximab)[136]. It is thus likely that further insight into the role of autoantibodies in PSC may be of clinical importance and further studies are warranted.

CONCLUSION

A large number of autoantibodies have been detected in PSC patients. The specificity of these antibodies is generally low and the frequencies vary largely between different studies. Interpretation of the literature is difficult because of small patient sample sizes and variable methodology for antibody detection. The presence of autoantibodies in PSC is often attributed to a nonspecific dysregulation of the immune system, but the literature in PSC points to the possible presence of specific antibody targets both in the biliary epithelium and in neutrophils. Further characterisation of such targets would probably yield important insight into the pathogenesis of PSC. The investigation of larger populations may also further define the role of autoantibodies in PSC as diagnostic tools.

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REVIEW

Epithelial-mesenchymal transition mediated tumourigenesis in the gastrointestinal tract

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Abstract

Epithelial-mesenchymal transition (EMT) is a highly conserved process that has been well characterised in embryogenesis. Studies have shown that the aberrant activation of EMT in adult epithelia can promote tumour metastasis by repressing cell adhesion molecules, including epithelial (E)-cadherin. Reduced intracellular adhesion may allow tumour cells to disseminate and spread throughout the body. A number of transcription proteins of the Snail superfamily have been implicated in EMT. These proteins have been shown to be over-expressed in advanced gastrointestinal (GI) tumours including oesophageal adenocarcinomas, colorectal carcinomas, gastric and pancreatic cancers, with a concomitant reduction in the expression of E-cadherin. Regulators of EMT may provide novel clinical targets to detect GI cancers early, so that cancers previously associated with a poor prognosis such as pancreatic cancer can be diagnosed before they become inoperable. Furthermore, pharmacological therapies designed to inhibit these proteins will aim to prevent local and distant tumour invasion.

Key words: Epithelial-mesenchymal transition; Transcription proteins; E-cadherin; Gastrointestinal cancer

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INTRODUCTION

Epithelial-mesenchymal transition (EMT) is a well-characterised embryological process that has been considered to play a vital role in tumour progression[1-5]. EMT has been shown to occur during gastrulation, as well as during the development of the neural crest, heart and the musculoskeletal system[6]. In the process of EMT, epithelial cells undergo a phenotypic switch to form mesenchymal cells that are similar in appearance to fibroblasts[2,3]. The change in cell type results in the loss of polarity and also the loss of tight intracellular adhesions maintained by epithelial cells *via* adherens junctions[1-3]. This is thought to allow dynamic cellular migration and increase embryogenic diversity[5]. However, *in vitro* evidence has outlined a role for the aberrant induction of EMT in adult epithelia during tumour metastasis[6,7]. In primary tumours, the induction of EMT can lead to structural changes involving cell adhesion molecules, and in particular epithelial-cadherin (E-cadherin)[8-10]. E-cadherin is a transmembrane glycoprotein that is localised in the adherens junction typically found in epithelial cells, and plays an important role in maintaining the structural integrity of epithelial sheets[11]. The loss of E-cadherin expression has been reported in several GI cancers including advanced colorectal carcinomas, oesophageal adenocarcinomas, gastric and pancreatic cancers[12-15]. Interestingly, experiments used to silence the expression of E-cadherin not only showed a morphological shift from an epithelial to a fibroblastoid phenotype, characteristic of EMT, but also a concomitant increase in invasive cell behaviour[16]. The loss of E-cadherin has been considered to augment cellular dissemination and tumour metastasis.

The mechanisms by which E-cadherin has been shown to be inactivated include gene mutations, promoter hypermethylation, chromatin remodelling,

post-translational modification and transcriptional repression[17-19]. The major proteins implicated in the transcriptional repression of E-cadherin include the zinc finger proteins Snai1 (Snail) and Snai2 (Slug), δEF (ZEB-1), Smad interacting protein 1 (SIP1) or ZEB-2, and a basic helix-loop-helix (bHLH) protein called Twist[20-22]. Snail was initially described in *Drosophila melanogaster* and was shown to be essential for dorsal-ventral patterning and mesoderm formation[23]. Knockout studies revealed that mice lacking the *Snail* gene died at gastrulation due to defective EMT[24]. *Snail* was found to down-regulate epithelial markers such as claudins, occludens, desmoplakin and cytokeratins, in addition to E-cadherin, and up-regulate mesenchymal markers including fibronectin and vitronectin during EMT[25,26]. Its homologue, Slug was discovered in developing chick embryos, and was found to be abundantly expressed in cells undergoing EMT in the primitive streak, neural crest and other mesenchymal tissue[26]. Over expression of Slug in mice induced the formation of mesenchymal tumours that were mainly leukaemias and sarcomas[27]. It was discovered that both Snail and Slug are able to bind directly to E-box motifs (CANNTG) on target gene promoters, and in particular the *CDH1* gene in order to down-regulate E-cadherin expression[20]. The zinc finger proteins ZEB-1 and SIP1 were also shown to be able to repress E-cadherin by binding to similar DNA sites as Snail on the E-cadherin promoter region[28]. Furthermore, microarray analysis revealed Twist as another strong candidate for the acquisition of invasive properties of tumour cells, although its mechanism of action is less clear[22].

ROLE OF E-CADHERIN REPRESSORS IN CANCERS OF THE UPPER GASTROINTESTINAL TRACT

Cancers of the upper GI tract, including oral, oesophageal and gastric tumours, are associated with significant mortality worldwide[29-31]. Tumours arising in the oral cavity are predominantly squamous cell carcinomas (SCC) in origin and have a tendency to spread rapidly[32]. Oral cancer accounts for around 197 000 deaths each year, throughout the world[33]. E-cadherin expression in normal oral mucosa, compared to pre-cancerous oral lesions and primary oral SCC has been shown to be sequentially lower in each of these stages, respectively[34]. Moreover, Snail-mediated repression of E-cadherin has been confirmed in oral SCC cell lines[35]. Further analysis of primary and recurrent oral SCC, showed that the over-expression of Snail in the primary oral SCC lead to ZEB-1 and SIP1 up-regulation with a concomitant loss of E-cadherin[36]. This expression profile, now matching that of the recurrent oral SCC, may suggest that Snail is able to regulate the function of other E-cadherin repressors such as ZEB-1 and SIP1 in oral SCC.

Repression of E-cadherin has also been reported for advanced human oesophageal cancers[37]. These tumours are known to be particularly aggressive and have a 5-year survival rate of around 8% in the United Kingdom[38]. Oesophageal cancer is histologically divided into squamous cell carcinomas and adenocarcinomas. Oesophageal SCC commonly arises in the upper third of the oesophagus and its predominant aetiological factors include alcohol and nicotine abuse[39]. Analysis of tissue samples from patients with oesophageal SCC suggests that Snail is associated with repressed E-cadherin expression in these primary tumours[40]. In addition, Slug has been shown to be over-expressed in primary oesophageal SCC, correlating with depth of tumour invasion, lymph node metastasis and poorer clinical outcome[41]. Similarly, evaluation of Twist in oesophageal SCC revealed significantly higher Twist expression relative to non-neoplastic tissue[42].

Conversely, oesophageal adenocarcinomas arise in the lower third of the oesophagus[39]. The strongest known risk factor for the development of oesophageal adenocarcinoma is the presence of Barrett's metaplasia[43]. This is a pre-malignant state that is characterised by the replacement of native squamous oesophageal epithelium by columnar cells, and is considered to occur secondary to prolonged reflux of gastric content in the lower part of the oesophagus[44]. A recent study by Jethwa *et al* has reported an over-expression of nuclear Slug in oesophageal adenocarcinoma relative to both normal squamous and Barrett's metaplasia specimens[45]. Interestingly, no such association was observed for both Snail and Twist[45,46].

Gastric carcinomas also form part of the upper GI cancers, and although the incidence of these tumours seems to be declining, they are still responsible for around 700 000 deaths per annum, worldwide[47]. According to Lauren's classification, gastric cancer can be subdivided into two morphologically distinct groups; diffuse and intestinal gastric cancers[48]. The aetiology of gastric cancer has been mainly linked to E-cadherin mutations, promoter hypermethylation and *H pylori* infection, but there is scarce literature on the role of E-cadherin repressors in these tumours[49,50]. Snail-regulated repression of E-cadherin has been reported for diffuse gastric cancer[25], and over-expression of Slug has been shown in both diffuse and intestinal gastric carcinoma[51]. Previous work has also shown that in diffuse gastric carcinomas, raised Twist expression correlates with reduced E-cadherin levels, whereas in intestinal gastric cancer, SIP1 is mainly associated with reduced expression of E-cadherin[25]. Interestingly, a strong correlation between neuronal-cadherin (N-cadherin) and Twist expression has been reported in diffuse-type gastric carcinoma[25]. The up-regulation of N-cadherin has been associated with an invasive tumour phenotype and is considered to over-ride the function of E-cadherin[52]. Therefore, it is suggested that Twist may be implicated in mediating a switch from E-cadherin to the N-cadherin, in order to increase tumour cell motility[25]. A more recent study has confirmed this by using human gastric carcinoma cell lines to show that the suppression of Twist leads to a loss of cellular migration as well as N-cadherin expression[53].

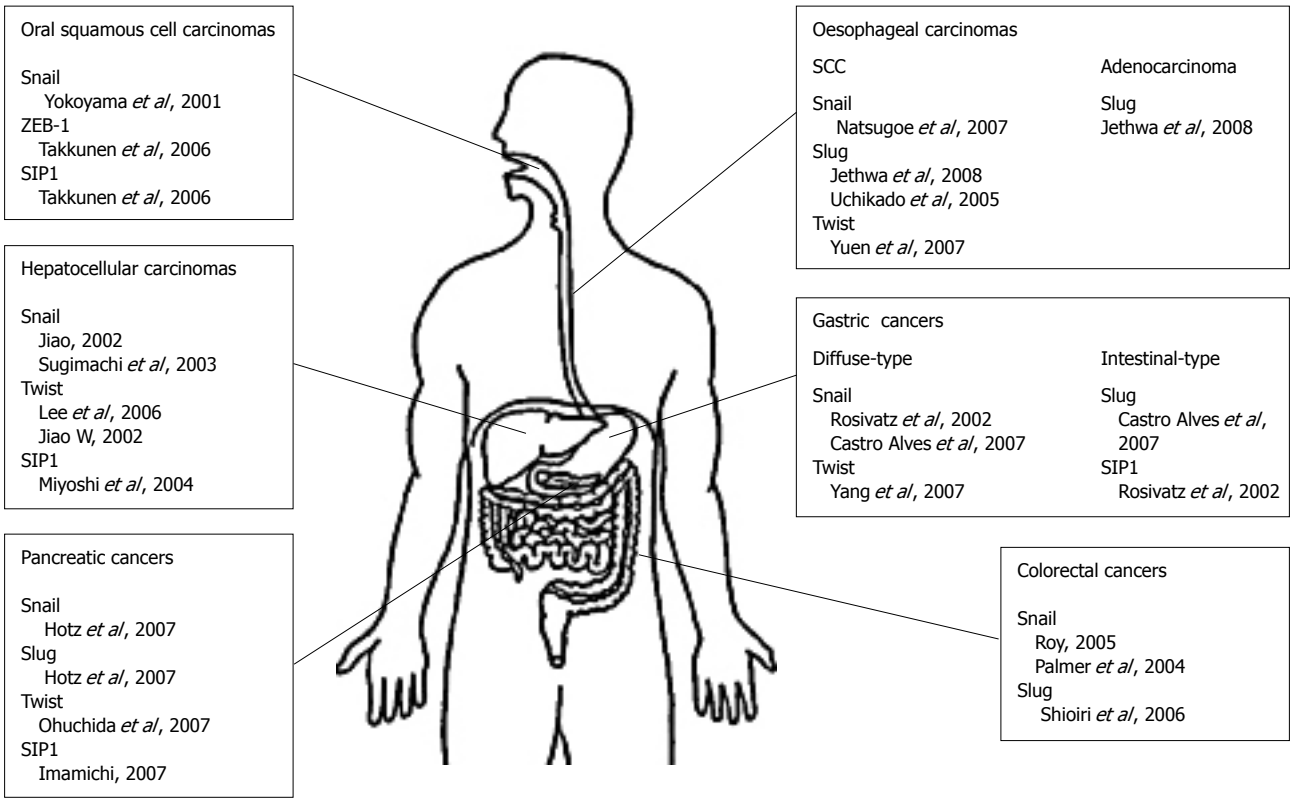

Figure 1 Evidence for the role of EMT regulators in gastrointestinal cancer.

COLORECTAL CANCER PROGRESSION

Colorectal cancer (CRC) is the third most common malignancy worldwide[54]. Its pathogenesis is characterised by clinical and histopathological changes known as the adenoma-carcinoma sequence, where normal colonic epithelium becomes hyper-proliferative and forms adenomatous polyps that progress to malignant disease[55]. The adenoma-carcinoma sequence is considered to occur as a result of sequential genetic changes involving defined oncogenes and tumour suppressor genes, as proposed by Fearon and Vogelstein in 1990[56]. Several studies have reported mutations of the tumour suppressor adenomatous polyposis coli (APC) gene in CRC[57-59]. These mutations are amongst the earliest genetic events found in the majority (up to 80%) of sporadic cases of CRC[60,61]. The main tumour suppressor function of APC has been shown to be in its ability to mediate the proteosomal degradation of intracellular β-catenin, a key member of the Wnt signalling cascade[62]. Physiologically, Wnt factors are able to induce the stabilization of cytosolic β-catenin, which then associates with T cell factor/lymphoid enhancer factor-1 (TCF) proteins in the nucleus to initiate the transcription of Wnt target genes[61-63]. These include genes such as c-myc, cyclin D1, Ephrin B2 and matrilysin[63]. In CRC, mutations of both APC and β-catenin (in 10% of cases) cause deregulation of intracellular β-catenin levels which leads to the nuclear accumulation of β-catenin[62]. This causes aberrant and constitutive expression of Wnt target genes, and thus the development of CRC[63]. Interestingly, β-catenin can also interact with the cytoplasmic domain of E-cadherin, providing a link to the actin cytoskeleton *via* its binding to α-catenin[64]. However, it is unclear whether the loss of this interaction with β-catenin, in more aggressive forms of CRC where E-cadherin is down-regulated, promotes TCF-dependent transcription[65].

Several mechanisms of E-cadherin repression have been reported in CRC, including gene mutations and promoter hypermethylation. However, transcriptional repression of E-cadherin and associated up-regulation of Snail is also considered to play a role in the progression of CRC[66-68]. Analysis of Snail in human CRC has shown that 78% of the tumour samples examined over-expressed this protein[68]. Slug expression has been shown to be positive in 37% of cases of primary CRC, which correlated significantly with metastatic spread of the cancer[69]. Evidence for ZEB-1, SIP1 and Twist-mediated repression of E-cadherin has not yet been established in CRC[70].

OTHER SOLID TUMOURS OF THE GASTROINTESTINAL SYSTEM

Pancreatic cancer confers possibly the worst prognosis of the GI cancers, since it presents very late[71,72]. Evidence suggests that both Snail and Slug are over-expressed in pancreatic tumours[72]. A recent study explored Twist expression in invasive ductal carcinoma (IDC) of the pancreas and its associated pre-malignant lesion intraductal papillary mucinous neoplasia (IPMN). Although it was limited by sample size, Twist expression

was found to be significantly higher in IDC compared to matched non-tumourous and IPMN samples[73]. SIP1 expression has also been shown to be raised in pancreatic tumours[74].

Studies have also reported the over-expression of Snail in Hepatocellular carcinomas (HCC)[75,76]. Twist expression has also been shown to be raised in HCC, which correlates with the metastatic potential of this type of tumour[77]. Miyoshi *et al* outlined the role of SIP1 in HCC by showing that transfection of SIP1 into HCC cell lines induced cellular dedifferentiation as well as E-cadherin repression. Vimentin and fibronection, along with various matrix metalloproteinases (MMPs), were also up-regulated, and it was hypothesised that MMP up-regulation augments SIP1-induced HCC progression[78].

CONCLUSION

EMT is a context dependent process that is considered to be involved in the progression of GI tumours[5-7]. Snail and associated repressors of E-cadherin have been implicated in EMT, and the evidence for their role in GI cancer is summarised in Figure 1. Transcriptional repressors of E-cadherin may be useful therapeutic targets for the prevention of local invasion and distant metastasis in GI malignancies. Inhibition of these proteins may also, for the first time, allow early detection of GI cancers associated with a fatal prognosis such as pancreatic cancer to enable early intervention and avoid the situation where this type of cancer becomes inoperable. The same principle can also be applied to other pre-malignant lesions such as Barrett's metaplasia to improve the management of oesophageal adenocarcinomas. However, it is important to consider that these transcriptional repressors of E-cadherin, or EMT regulators, also have other cellular functions. Both Snail and Twist have been associated with anti-apoptotic functions and Snail has also been implicated in cell adhesion and migration[79,80]. Furthermore, some evidence suggests that the expression of E-cadherin is higher in metastatic foci, such as in CRC, thus the reverse process of EMT, or mesenchymal-epithelial transition (MET), may be required in the formation of distant metastases[8]. Therefore, the grade and location of different GI cancers will also need to be considered before commencing any pharmacological treatment targeting regulators of EMT.

Future studies should also consider the various signalling molecules that activate EMT, including the Epidermal growth factor (EGF) family members, Fibroblastic growth factors (FGF), Insulin-like growth factors (IGF), bone morphogenic proteins (BMP) and Wnt factors[79]. It is necessary to explore the interactions between these molecules, their signalling pathways, and the Snail super-family of proteins in GI tumours. This may allude to novel combined treatment regimes to improve clinical outcome. Whilst it is clear that Snail and associated regulators of EMT are implicated in GI carcinogenesis, the role of EMT in cancer is further complicated by the fact that a number of novel EMT regulators have been identified, including molecules such as MMP-3, Met, Goosecoid, Kaiso, TGF-β, FOXC2, GSK3β, Smad-3, Pez and ILK[4]. Additional research is required to support the growing literature regarding the process of EMT, in order gain full insight into its role in GI cancer progression.

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Endoscopic stenting-Where are we now and where can we go?

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Abstract

Self expanding metal stents (SEMS) play an important role in the management of malignant obstructing lesions in the gastrointestinal tract. Traditionally, they have been used for palliation in malignant gastric outlet and colonic obstruction and esophageal malignancy. The development of the polyflex stent, which is a removable self expanding plastic stent, allows temporary stent insertion for benign esophageal disease and possibly for patients undergoing neoadjuvant chemotherapy prior to esophagectomy. Potential complications of SEMS insertion include perforation, tumour overgrowth or ingrowth, and stent migration. Newer stents are being developed with the aim of increasing technical and clinical success rates, while reducing complication rates. Other areas of development include biodegradable stents for benign disease and radioactive or drug-eluting stents for malignant disease. It is hoped that, in the future, newer stents will improve our management of these difficult conditions and, possibly, provide prognostic as well as symptomatic benefit in the setting of malignant obstruction.

Key words: Endoscopy; Stent; Palliation; Bowel obstruction; Malignancy

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INTRODUCTION

Self expanding metal stent (SEMS) insertion has an important role in the management of malignant gastrointestinal obstruction. There are several types and sizes of SEMS on the market. Each has its own characteristics in terms of radial forces exerted, foreshortening on deployment, and flexibility. SEMS are made of either stainless steel [e.g. Z-stent (Cook)] or alloys such as Nitinol [e.g. Ultraflex (Boston Scientific), Alimaxx E (Alveolus)] or Elgiloy [e.g. Wallstent (Boston Scientific)][1]. Stent insertion is also increasingly used in benign esophageal disease, such as non-malignant strictures and anastomotic leaks. The Polyflex stent (Boston Scientific) is a self expanding plastic stent which has been approved for use in the management of benign and malignant esophageal strictures.

Enteral SEMS, i.e. for the duodenum and colon, are generally inserted through the scope (TTS). These are deployed over a guidewire under direct vision, usually with fluoroscopic guidance. Esophageal stents are not TTS and are deployed under fluoroscopic guidance after delineating the margins of the stricture endoscopically.

In this article, we review the current state of play with respect to enteral and esophageal stents, the latest developments, and possible future directions.

ESOPHAGEAL STENTING

SEMS have been in use for malignant dysphagia and trache-esophageal fistulae (TEF) since the early 1990s when they replaced rigid plastic stents. They are relatively easy to deploy, have a high technical success rate and provide rapid relief of dysphagia[2]. However, insertion of SEMS has a complication rate of 26%-52%[3-7] with 1 in 6 requiring further stents[8]. Procedure related mortality is 2%-3%[7,8]. Complications associated with esophageal stent insertion include perforation, bleeding,

stent migration, reflux, chest pain, recurrent dysphagia due to tumour overgrowth or ingrowth, migration, and food bolus impaction. Although SEMS insertion is still the treatment of choice for TEF, it appears not to be the safe, one-off treatment for malignant dysphagia that it was once hoped to be.

Comparisons between SEMS and brachytherapy for esophageal malignancy have shown improved dysphagia scores at 30 d with reduced complications[9] and improvements in quality of life, dysphagia, and eating scales[10] for brachytherapy. It has been suggested that, as stent re-intervention is likely to be increased for those who live longer, SEMS should be considered for those with a poorer prognosis, and chemo/radiotherapy, with temporary stent placement, for those with a longer life expectancy[2]. A Korean group inserted a removable nitinol stent in 47 patients who had concurrent radiotherapy and extracted the stent in 24 patients after 4 wk, leaving the stent in place in the remaining patients[11]. The complication and re-intervention rates were significantly lower in the group in which the stent was extracted, while the dysphagia-progression-free and overall survival rates were significantly longer. No randomized trials have yet been conducted with the Polyflex stent, which is the only removable stent licensed in the USA in this setting. Further randomized trials of SEMS in combination with other treatment modalities would help determine the optimal management strategy in terms of symptom control and overall survival. Drug-eluting and radioactive stents may also have a future role in the management of esophageal malignancy; these have been tested with success in animal models[12,13].

In cases where the distal margin of the stent crosses the gastro-esophageal (GE) junction there are now SEMS available with an anti-reflux mechanism. Survival has been shown to be reduced in patients in whom the stent crossed the GE junction[14]. A study which compared an open stent with the Z-stent with Dua antireflux valve found that 96% of patients with the open stent had reflux symptoms, compared with 12% with the antireflux mechanism[15]. Several other SEMS with antireflux mechanisms have been manufactured. Further work will be required to determine the overall efficacy and complication rates of these stents for distal esophageal and cardia tumours.

Many of the available SEMS are covered to reduce the risk of tumour in-growth and to seal TEF. As the risk of stent migration is higher with covered stents, many have flared ends and uncovered segments at both ends to anchor on to the tissue. Fully covered SEMS may prove useful in benign disease as they are potentially removable but further experience in this area is required.

The Polyflex stent is the only stent currently licensed for benign disease but there has been an interest in the development of biodegradable stents. These would theoretically exert their effect before slowly breaking down and subsequent stent extraction, which can be stressful for the patient and physician, is avoided. A small case series from Japan had promising results when a biodegradable stent constructed from poly-l-lactic acid monofilaments was used to treat benign esophageal stenoses[16].

GASTRIC OUTLET OBSTRUCTION (GOO) AND DUODENAL STENTING

Stent placement for GOO was first described in 1992[17]. Patients with GOO are generally very ill and in the terminal phase of a malignant process. Gastrojejunostomy (GJJ) has traditionally been the procedure of choice for GOO. However, insertion of a SEMS for GOO offers a relatively safe and much less invasive alternative to gastrojejunal bypass. Most trials comparing GJJ with SEMS insertion for GOO are prospective or retrospective comparative studies or case series evaluating either SEMS insertion or GJJ. A summary of the prospective and larger retrospective case series is given in Table 1. A more recent comprehensive review of stent insertion *versus* GJJ for GOO included a total of 1046 patients undergoing stent insertion and 297 undergoing GJJ[39]. There was no difference between SEMS insertion and GJJ in terms of technical success (96% *vs* 100%), early (7% *vs* 6%) and late (18% *vs* 17%) major complications, or persisting symptoms (8% *vs* 9%). Initial symptom relief was higher for SEMS (89% *vs* 72%). Recurrent obstructive symptoms were higher for SEMS (18% *vs* 1%) but hospital stay was shorter (13 d *vs* 7 d) with a mean survival of 105 d after stent placement and 164 after GJJ. These results suggest that stent placement may be the preferred option for patients with a shorter life expectancy but GJJ is preferable for patients with a more favourable prognosis.

Several stents are available for gastroduodenal use including the Wallstent Enteral, Wallflex Enteral Duodenal (Boston Scientific), Choo stent (Solco Intermed Co. Ltd. and Mi Tech Co. Ltd), and the Song stent (Stentech). The aim of stent manufacturers is to produce a SEMS which is easy to insert, is clinically effective and carries a low complication and migration rate. The use of the new Nitinol Wallflex stent was investigated by Van Hooft *et al*[40] who inserted a total of 66 Wallflex stents in 62 patients. with a clinical success rate of 85%. Median hospital stay was 6 d, and 10 of 60 patients (17%) who had follow up data for 30 d developed complications. They concluded that the new stent was effective and relatively safe.

Other recently developed stents include the Niti-S enteral stent (Taewoong Medical Co.) which has a woven rather than the usual braided design, leading to improved flexibility and reduced foreshortening and, it is hoped, reduced migration, as well as dual stents (e.g. Niti-S Comvi, Taewoong Medical Co., and the dual expandable nitinol stent, S&G Biotech). These have a covered layer to reduce tumour ingrowth and an uncovered layer to reduce migration. These newer stents have shown promising results in case series[32,33,37] but randomized comparisons with conventional stents are required to further assess their efficacy.

Table 1 Summary of case series of SEMS placement for gastric outlet obstruction (%)

Authors	Yr	Study design	*n*	Technical success	Clinical success	Major complications (early and late)
de Baere *et al*[18]	1997	Prospective	10	100	94	28
Bethge *et al*[19]	1998	Prospective	6	100	100	33
Jung *et al*[20]	2000	Prospective	19	95	100	26
Pinto Pabon *et al*[21]	2001	Prospective	31	100	90	10
Kim *et al*[22]	2001	Prospective	29	90	96	29
Lopera *et al*[23]	2001	Prospective	16	94	81	19
Profili *et al*[24]	2001	Prospective	15	100	93	14
Lee *et al*[25]	2001	Prospective	11	87	82	0
Espinel *et al*[26]	2001	Prospective	6	100	100	0
Jung *et al*[27]	2002	Prospective	39	97	95	36
Jeong *et al*[28]	2002	Prospective	18	100	94	28
Schiefke *et al*[29]	2003	Prospective	20	100	100	nr
Holt *et al*[30]	2004	Prospective	28	93	93	21
Huang *et al*[31]	2007	Prospective	14	100	86	14
Kim *et al*[32]	2007	Prospective	213	94	94	21
Lee *et al*[33]	2007	Prospective	11	100	91	18
Lowe *et al*[34]	2007	Prospective	87	97	87	10
Maetani *et al*[35]	2007	Prospective	37	97	94	19
Song *et al*[36]	2004	Retrospective	102	99	84	9
Telford *et al*[37]	2004	Retrospective	176	97	84	9
Bessoud *et al*[38]	2005	Retrospective	72	97	90	15

nr: Not reported.

Increasingly innovative techniques for stent insertion are also being pioneered. The development of double balloon enteroscopy has allowed us to perform therapeutic procedures in areas that were previously beyond our reach. Ross *et al* successfully inserted a SEMS in the distal duodenum for a patient with metastatic lung cancer using double balloon enteroscopy[41]. This raises the possibility of stent insertion in patients with a single point of malignant small bowel obstruction that is beyond the reach of conventional endoscopes.

COLONIC STENTING

The use of SEMS in the palliation of malignant colonic obstruction was first described in 1991[42]. The current stents available are uncovered but there have been reports on the use of uncovered and covered esophageal stents in the colon. Overall technical success rates are generally in excess of 95% with relief of obstructive symptoms in 85%-90% for palliative stenting[1]. In a comprehensive review of 58 publications on colorectal stent publications from 1990 to 2000[43] stent insertion was successful in 551 of 598 cases (92%). There was a 4% rate of perforation, 10% migration rate and 10% re-obstruction rate. Stent migration was associated with laser pre-treatment, concurrent chemotherapy, covered stent use, and benign disease. The perforation rate was higher in the studies in which balloon pre-dilation was performed (10% *vs* 2%), suggesting that this should not be performed routinely. A variety of stents were used in the different studies but most of them were uncovered. One study, which used partially or fully covered stents, had a migration rate of 22%[44].

Many earlier series used esophageal stents for colonic stenting and it is hoped that specifically designed colorectal stents will have lower rates of migration. For example, in a prospective study with 44 patients[45] the nitinol Ultraflex precision colonic stent migrated in one patient (2%) who had commenced chemotherapy shortly after stent insertion. There was a technical success rate of 95% and a 6 mo clinical success rate of 81%. There have been very few comparisons between different stent types. A small retrospective study comparing the Ultraflex stent and the Wallstent found that they both provided adequate relief of obstruction but the Ultraflex had a significantly lower rate of delayed complications, need for re-intervention, and a non-significant reduction in early migration and occlusion[46].

In recent years there has been a move towards SEMS insertion as a "bridge" to surgery for patients who present with acute malignant obstruction. In the event that a patient is subsequently deemed unsuitable for a curative resection, the stent provides palliation. 10%-30% of patients with colonic cancer present with obstructive symptoms[47] and in many centres surgical decompression remains the primary management option for such patients, either due to local preference or resources. Morbidity and mortality rates have been quoted at 32%-64% and 15%-34%, respectively, for patients who undergo emergency surgery[48-52]. Up to 40% of these patients are left with a permanent colostomy[53]. For those patients who are subsequently found to have operable disease they then need a second surgical procedure. Stent insertion is appealing as it allows these patients to have adequate rehabilitation and preparation before an elective procedure, while avoiding invasive surgery for palliative patients.

Martinez-Santos *et al*[50] performed a prospective study investigating the results of colonic stenting *versus* emergency surgery in 72 patients presenting with left

sided malignant colorectal obstruction. Forty-three patients had preoperative stent insertion followed by elective surgery (if necessary) and 29 had emergency surgical treatment. Surgical resection was subsequently found not to be indicated in 18 of the patients who had SEMS insertion and in 3 from the control group. SEMS insertion was clinically successful in 41 cases (95%). Of those patients with colonic stents who proceeded to surgery 85% had a primary anastomosis, compared to 41% in the non-stent group ($P = 0.0025$), with a lower need for a colostomy (15% *vs* 59%). There were also significantly reduced severe complications, re-intervention rates, total hospital stay, and ICU stay. Overall, stent placement prevented 17 of 18 (94%) unnecessary operations. In a long-term follow up study there was no difference in 3 years (48% *vs* 50%) and 5 years (40% *vs* 44%) survival in the SEMS and emergency surgery groups respectively, but post-operative complications were significantly lower in the stent group[51]. Therefore, stent insertion as a bridge to surgery is technically and clinically successful, relatively safe, and cheaper than emergency surgery for patients who present with malignant left sided obstruction.

SEMS insertion is not currently approved for benign disease of the colon, primarily because of high failure and complication rates, as well as an inability to remove the stent endoscopically[54]. In one study there was a failure rate of 63% for 8 patients[55]. In the largest series to date 23 patients had an SEMS placed for benign colonic disease[56]. There was a 100% technical success rate and 95% (22/23) clinical success rate. Eight of the 23 patients (38%) had major complications, 7 of which (87%) occurred within the first week. Sixteen of the 19 patients who underwent a colectomy were successfully converted from an emergency procedure to an elective one; 8 patients did not require a colostomy. SEMS insertion should probably not be considered as a definitive treatment option for benign colonic strictures, in view of the high rate of complications in the limited published data available. However, in the setting of colonic obstruction it may be appropriate as a temporary measure to facilitate decompression with subsequent elective surgery, rather than proceeding to emergency colectomy.

CONCLUSION

Endoscopic stenting remains an important tool in the management of malignant conditions of the esophagus, gastric outlet, and colon. As newer stents are developed, randomised trials are required to determine which provides the most benefit. For esophageal malignancy the evidence suggests that SEMS insertion may be appropriate for patients with a poorer prognosis, with temporary SEMS and chemoradiotherapy for those with a longer life expectancy. However, further trials are required to clarify the optimum management of these patients. For patients undergoing neo-adjuvant therapy prior to esophagectomy, a small retrospective trial reported favourable results for temporary placement of the Polyflex stent[57]. The Polyflex stent is being increasingly used for benign esophageal strictures and has also been used with success in the management of post-operative esophageal leak[58]. Another novel use for temporary esophageal stent placement was in the management of acute esophageal variceal bleeding[59]. Although the findings of this small study were positive, a large comparative trial would be required before SEMS could replace the current therapy for bleeding varices.

Colonic stenting should be considered for palliation in malignant obstruction and as a bridge to surgery in the setting of acute obstruction. The results of further randomized controlled trials, such as the Dutch Stent-in 2 study[60], are awaited to bolster the existing evidence. Covered stents and pre-deployment dilatation appear to increase the complication rate. The development of stents with longer delivery systems will hopefully make the right colon more accessible also. Double balloon enteroscopy may also allow stent insertion in areas that were previously beyond our reach.

As stenting devices and our skills develop, endoscopic capabilities will continue to expand. Bioabsorbable stents may allow a safe and effective method of temporary stent placement, without the need for a further procedure. Radioactive and drug-eluting esophageal stents have already been trialled in animal models; it is hoped that such stents in the future will have prognostic as well as symptomatic benefit for patients with malignant obstruction. We await these new developments with anticipation.

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GASTRIC CANCER

PCR-SSCP-DNA sequencing method in detecting *PTEN* gene mutation and its significance in human gastric cancer

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Abstract

AIM: To discuss the possible effect of *PTEN* gene mutations on occurrence and development of gastric cancer.

METHODS: Fifty-three gastric cancer specimens were selected to probe *PTEN* gene mutations in genome of gastric cancer and paracancerous tissues using PCR-SSCP-DNA sequencing method based on microdissection and to observe the protein expression by immunohistochemistry technique.

RESULTS: PCR-SSCP-DNA sequencing indicated that 4 kinds of mutation sites were found in 5 of 53 gastric cancer specimens. One kind of mutation was found in exons. AA-TCC mutation was located at 40bp upstream of 3′ lateral exon 7 (115946 AA-TCC). Such mutations led to terminator formation in the 297th codon of the *PTEN* gene. The other 3 kinds of mutation were found in introns, including a G-C point mutation at 91 bp upstream of 5′ lateral exon 5(90896 G-C), a T-G point mutation at 24 bp upstream of 5′ lateral exon 5 (90963 T-G), and a single base A mutation at 7 bp upstream of 5′ lateral exon 5 (90980 A del). The PTEN protein expression in gastric cancer and paracancerous tissues detected using immunohistochemistry technique indicated that the total positive rate of PTEN protein expression was 66% in gastric cancer tissue, which was significantly lower than that (100%) in paracancerous tissues ($P < 0.005$).

CONCLUSION: *PTEN* gene mutation and expression may play an important role in the occurrence and development of gastric cancer.

Key words: Gastric cancer; *PTEN* gene; PCR-SSCP; DNA sequencing; Mutation

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INTRODUCTION

The occurrence and development of gastric cancer, like other malignant tumors, are a complicated process involving participation of polygene and many factors[1-4]. It is generally considered that protein tyrosine phosphatase level plays an important role in the process. Mutation of the *PTEN* gene encoding for protein tyrosine phosphatase and abnormal expression of protein are significantly correlated with the occurrence and development of malignant tumors such as glioblastoma, prostate cancer, malignant melanoma, and breast cancer, *etc*[5-11]. However, only few studies are available on *PTEN* gene mutation and protein expression in gastric cancer[3,12-15]. The aim of this study was to detect the *PTEN* gene mutation in gastric cancer and paracancerous tissue from 53 patients by PCR-SSCP-DNA sequencing method and to observe the protein expression by immunohistochemistry technique in order to find the effect of *PTEN* gene on the occurrence and development of gastric cancer.

MATERIALS AND METHODS

Objects

Fifty-three gastric cancer and corresponding paracancerous normal tissue samples were obtained at surgery. All the samples were formalin fixed, paraffin embedded, and pathologically confirmed. Of the 53 patients, 41 were males and 12 were females with a mean age of

Table 1 Primer sequence, length and annealing temperature of exons 5-8 in *PTEN* gene

	Primer sequence	Primer length (bp)	Amplification fragment length (bp)	Annealing temperature (℃)
Exon-5F:	ACCTGTTAAGTTTGTATGCAAC	22	379	52
R	TCCAGGAAGAGGAAAGGAAA	20		
Exon-6F:	CATAGCAATTTAGTGAAATAACT	23	274	52
R	GATATGGTTAAGAAAACTGTTC	22		
Exon-7F:	TGACAGTTTGACAGTTAAAGG	21	263	58
R	GGATATTTCTCCCAATGAAAG	21		
Exon-8F:	CTCAGATTGCCTTATAATAGTC	22	558	52
R	TCTGTTACTTGCTACGTAAAC	21		

65.6 years, ranging 39-81 years. The tumor diameter was greater than 3 cm in 37 patients. The tumor was located in gastric antrum of 30 patients, in gastric body of 16 patients, and in gastric cardia of 7 patients, respectively. Invasion was restricted in mucosa and submucosa of 2 patients (Ⅰ), in muscular layer of 12 patients (Ⅱ), in chorion and subchorion of 15 patients (Ⅲ), in neighboring organs of 24 patients through chorion (Ⅳ). Lymph node metastasis was found in 32 patients, distant metastasis in 8 patients, embolization in 45 patients. Well-differentiated tumor was found in 1 patient, moderately-differentiated tumor in 35 patients, and poorly-differentiated tumor in 27 patients. pTMN stage Ⅰ was identified in 13 patients, stage Ⅱ in 6 patients, stage Ⅲ in 26 patients, and stage Ⅳ in 8 patients.

Reagents

Tris base, EDTA, $2H_2O$-Na_2, Taq DNA polymerase and Taq Ⅰ were purchased from Shanghai Sangon Biological Engineering Technology and Service Co. Ltd. SDS was purchased from AMRESCO Inc. Protein enzyme was purchased from Jingmei Biotechnology Co. Ltd. dNTPs was purchased from Pharmacia Inc. Polyclonal rabbit anti-PTEN antibody and immunohistochemistry staining reagent kit (Rabbit SP Kit) were purchased from Zymed Laboratories Inc. DAAB kit and citrate buffer were purchased from Beijing Zhongshan Biotechnology Co. Ltd. Phosphate-buffered saline (PBS) was purchased from Fuzhou Maixin Biotechnology Co. Ltd.

Genome DNA extraction from paired gastric cancer and paracancerous tissues

Three paraffin slices (7 μm) were dried in a galvano-thermy box at 60℃ for 30 min, hydrated in gradient ethanol after deparaffinized in dimethylbenzenel, adequately rinsed with tap water and naturally dried. Necrotic tissues were removed under inverted microscope and no carcinoma cells were found in paracancerous tissues. Gastric cancer and paracancerous tissues were put into a 1.5 mL eppendorf tube into which 50 μL digest buffer solution was added. The tube was overturned several times to blend it adequately, bathed in water for 8 h at 65℃ and shaken several times. Protein enzyme k was deactivated at 95℃ for 8 min and then centrifuged at 10000 r/min for 10-15 min. Transfer supernatant, namely genome DNA, was transferred to another antisepsis tube and stored at 4℃ for application.

PCR amplification of sequence of exons 5-8 in PTEN gene

PCR system is composed of 5 μL PCR buffer solution,5 μL dNTP (2.5 mmol/L), 2 μL primer (F) (10 pmol/μL), 2 μL primer (R) (10 mmol/L), 2 μL DNA template, 1 μL Taq DNA polymerase (5 units/μL), 33 μL ddH_2O. PCR conditions were at 94℃ for 4 min × 1 cycle, at 94℃ for 30 s, at 52℃ (fifth, sixth and eighth exons) at 58℃ (seventh exon) for 30 s, at 72℃ for 30 s × 30 cycles, at 72℃ for 7 min × 1 cycle. Five μL of the PCR amplified product was put on a 2% agarose gel containing 0.5 g/L EB, 100 bp DNA ladder as a standard reference, electrophoresed for 45 min at 100 V. The results were observed with an ultraviolet transmission reflect analysis instrument and photo was taken with an automatic gel documentation system. Primers used for detecting the mutation of exons 5-8 in the *PTEN* gene are listed in Table 1.

Enzyme cut reaction with Taq Ⅰ

Enzyme cut reaction is composed of 3.2 μL ddH_2O, 1.5 μL buffer Taq I, 10.0 μL PCR, 0.3 μL Taq I (10 unit/μL), and a total volume of 15.0 μL. The mixture was centrifuged for 15 s and heated for 3.5 h at 65℃. Ten μL enzyme cut product was put on a 2% agarose gel containing 0.5 g/L EB, 100 bp DNA ladder as a standard reference, electrophoresed for 45 min at 100 V. The results were observed with an ultraviolet transmission reflect analysis instrument and photo was taken with an automatic gel documentation system to evaluate the enzyme cut reaction.

SSCP analysis

Eight percent neutral polyacrylamide gel electrophoresis was performed as previously described[16]. In brief, 3 mL 40% acrylamide solution, 3 mL 5 × TBE solution, 3 mL 50% glycerin, 6 mL ddH_2O, 75 μL 10% ammonium persulfate, 7 μL TEMED, were blended adequately and poured into the gel, then concreted for 1 h at room temperature. Four μL PCR product (eighth exon enzyme cut product of exon 8) and 6 μL formamide sample were mixed. The mixture was centrifuged for 15 s, denatured at 95℃ for 10 min, bathed in ice for 10 min, put on an 8% neutral polyacrylamide gel, and electrophoresed with 1 × TBE buffer for 8 h at 14℃ and 300 V. The fixation

solution was infused into a flat utensil, into which gel was immerged, vibrated for 10 min, and washed 3 times (2 min each time) with ddH_2O. The gel was immerged into a staining solution, vibrated for 10 min, washed 3 times (20 s each time) with ddH_2O. The gel was then immerged into a display solution, vibrated until the sample signal became brown and the background became transparent yellow, and rinsed with tap water to stop display. The staining results were observed and photographs were taken.

According to the PCR-SSCP results of genome DNA, the difference in the single strand strip number and electrophoresis transference location, also known as the mobility shift, was considered PCR-SSCP positive.

DNA sequencing

Genome DNA from positive PCR-SSCP samples was amplified again in 80 μL reaction system. The product was identified by electrophoresis for bidirectional DNA sequencing.

Immunohistochemical staining

PBS was used instead of the primary antibody for blank and normal non-immunized rabbit serum was used instead of the primary antibody for negative. Following the specifications provided with the SP staining reagent box, the deparaffinized tissues were cut into 5 μm thick sections, washed 3 times (5 min each time) with PBS, incubated at room temperature in 3% H_2O_2 to eliminate the endogenous peroxidase activity, then wash additional 3 times (3 min each time) with PBS. Antigens were repaired with microwave (citrate buffer pH 6.0), naturally refrigerated to room temperature, washed 3 times (3 min each time) with PBS, incubated at room temperature with normal non-immunized serum solution for 15 min to indicate the non-specific sites, then incubated at room temperature with the primary antibody solution and horse radish peroxidase (HRP) tagged streptavidin for 15 min respectively, washed 3 times (3 min each time) with PBS. DAE stain was rinsed with PBS for 3 min, counter stained with hematoxylin for 1 min, rinsed with tap water for 2 min, differentiated with 1% hydrochloric ethanol, rinsed with tap water for 5 min, dehydrated with gradient alcohol, transparentized with dimethylbenzene. The sections were coated with neutral balata.

Criteria for positive PTEN protein immunohistochemical staining

Ten high power fields (50-300 cells/HP) were randomly selected for each section to measure histology (H) scores according to the percentage (P) and intensity (I) scores of positive cells ($H = P \times I$, P: percentage lower than 10% for score 0, 11%-40% for score 1, 41%-70% for score 2, and higher than 71% for score 3. I: intensity null for score 0, weak (faint yellow) for score 1, moderate (yellow) for score 2, strong (brown) for score 3. H measurement: score 0 or 1 for negative, score 2 or more for positive).

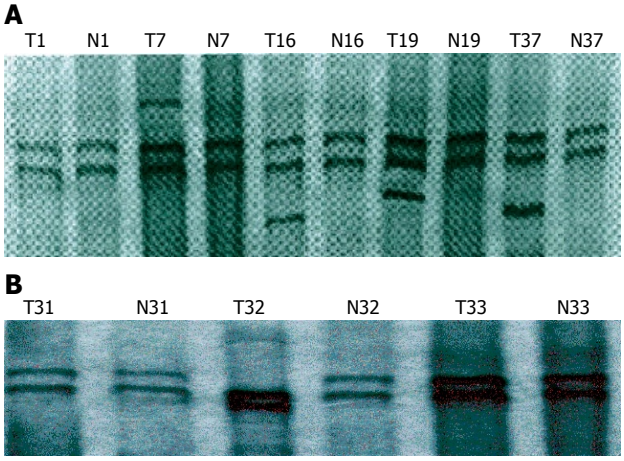

Figure 1 PCR-SSCP showing exons 5 (**A**) and 7 (**B**) in *PTEN* gene. T7, T16, T19 and T37: Positive SSCP; T1: Negative SSCP; T32: Positive SSCP; T31 and T33: Negative SSCP. N: Paracancerous tissue samples; T: Gastric cancer tissue samples.

Statistical analysis

Fisher's exact probability and chi-square test were used in statistical analysis. $P < 0.05$ was considered statistically significant.

RESULTS

Mutation of exons 5-8 in PTEN gene

Detection of the *PTEN* gene exons 5-8 of genome DNA in 53 paired gastric cancer and paracancerous tissue samples indicated that the amplified PCR product had no gene homozygous alteration and no large and/or alteration in the alleles.

Ten μL reaction product of PCR amplified exon 8 and Taq I enzyme cut reaction on a 2% agarose gel containing 0.5 g/L EB, 100 bp DNA ladder were used as a standard reference. The results indicated that the number and size were in accordance with the theory. The 281 bp, 247 bp, 30 bp segments were relatively justified as the complete enzyme cut reaction.

SSCP detection

In terms of mutation of exons-5-8 in the *PTEN* gene, positive PCR-SSCP was considered abnormal single strand number and mobility location. Of the 53 gastric cancer tissue samples, mutation occurred in 5 samples, the mutation rate was 9.4%. A surplus shift strip of exon 5 was found in 4 samples, the mutation rate was 7.5% (Figure 1A). Abnormal motility velocity (single strand strip mobility location) was observed in 1 sample at exon 7, the mutation rate was 1.9% (Figure 1B). There was no abnormal SSCP strip in exons 6 and 8.

DNA sequencing

Genome DNA from positive PCR-SSCP samples was amplified for bidirectional DNA sequencing. The results indicated that only one mutation was found in exons. As in the sample, AA-TCC mutation was located at

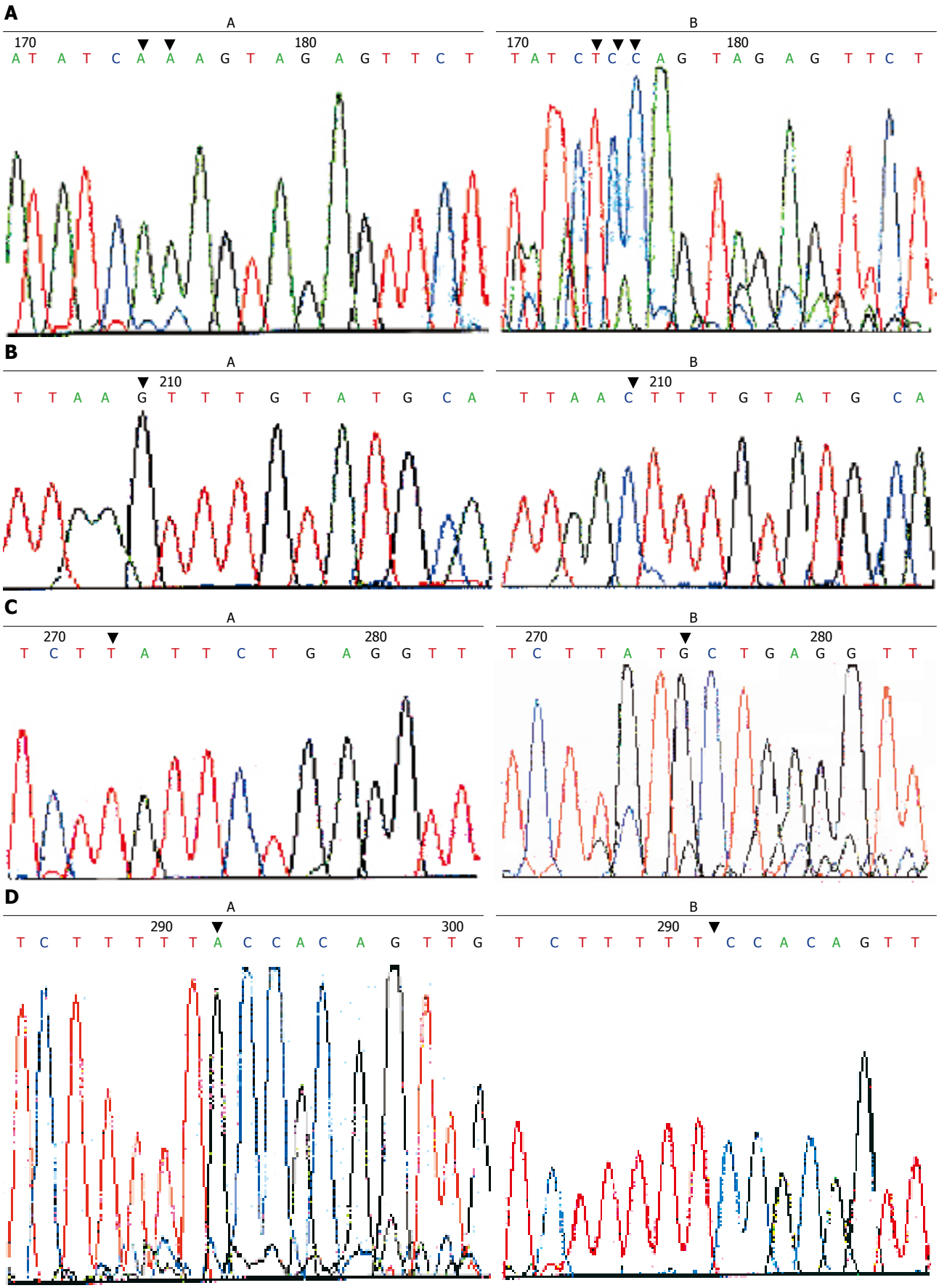

Figure 2 AA-TCC mutation at 40 bp upstream of 3' lateral in exon 7 (**A**), G-C point mutation at 91 bp upstream of 5' lateral exon 5 (**B**), T-G point mutation at 24 bp upstream (**C**) and single base A mutation at 7 bp upstream (**D**) of 5' lateral exon 5 in paired paracancerous and gastric cancer tissue samples.

Table 2 PTEN protein expression in gastric cancer and paracancerous tissue samples and intensity distribution *n* (%)

Clinicopathological parameters	Cases	PTEN protein expression				*P*
		-	+	++	+++	
Paracancerous	53	0 (0.0)	10 (18.9)	18 (34.0)	25 (47.2)	< 0.005
Gastric cancer	53	18 (34.0)	17 (32.1)	15 (28.3)	3 (5.7)	
Differentiation extent						< 0.005
Moderate and high differentiation	26	4 (15.4)	6 (23.1)	10 (38.5)	6 (23.1)	
Low differentiation	27	14 (51.9)	8 (29.6)	3 (11.1)	2 (7.4)	

40 bp upstream of 3' lateral in exon 7 (Figure 2A). Such mutations led to terminator formation in codon 297 of the *PTE*N gene. The other 3 kinds of mutation were found in introns, including a G-C point mutation at 91bp upstream of 5' lateral in exon 7 (Figure 2B), a T-G point mutation at 24 bp upstream of 5' lateral in exon 5 (Figure 2C), single base A mutation was deleted at 7 bp upstream of 5' lateral in exon 5 (Figure 2D).

PTEN *protein expression in gastric cancer and paracancerous tissues*

The PTEN protein was expressed in gastric cancer and paracancerous tissue samples. The expression of PTEN protein in gastric cancer samples was 66.0% and 100% in gastric cancer and paracancerous tissue samples, respectively ($P < 0.005$, Table 2).

Correlation between PTEN *protein expression and clinicopathological parameters in gastric cancer patients*

PTEN expression was not significantly correlated with the clinicopathological parameters in gastric cancer patients, such as gender and age of the patients, location and size of the carcinoma, distant metastasis, and embolization ($P > 0.05$), but was significantly correlated with infiltrating depth, lymph node metastasis, and pTMN staging ($P < 0.05$, Table 3). There was also a significant difference between the moderate and high differentiation groups ($P < 0.005$, Table 1).

DISCUSSION

Protein tyrosine phosphatase level, one of the multi-factors interacting in the period of normal cell growth and division, is determined between protein tyrosine kinase and protein tyrosine phosphatase. The imbalance between the two enzymes affects cell signal transference and cell division, thus leading to malignance of the cells. The occurrence and development of gastric cancer, as other malignant tumors, are an uncontrolled growth and differentiation process of multi-factors involving participation of many genes, including mutation and/or low expression of tumor suppressor gene. At present, researches on structure alteration of tumor suppressor genes in tumor tissues and tumor cell lines, including point mutation, deletion, insertion, cut point, *etc*, indicate that the mutation rate of tumor suppressor genes is 33%-50% in endometrial cancer, 25% in glioblastomas, 21% in ovarian cancer, 13% in prostate cancer, less than 5% in breast and thyroid cancer[5-11].

It was reported that PTEN protein expression is decreased in normal gastric mucosa, intestinal metaplasia, dysplasia and gastric cancer, which is significantly higher in normal gastric mucosa and intestinal metaplasia than in dysplasia and gastric cancer[17,18].

In order to identify the exact role of *PTE*N mutations in occurrence and development of gastric cancer, we used PCR-SSCP-DNA sequencing technique to isolate cancer cells from non-cancer cells to study the sequences of exons 5-8 and certain introns which are frequently mutated. The results indicate that the total mutation rate was 9.4% (5/53), with 3 mutations in introns, including a G-C point mutation at 91 bp upstream, a T-G point mutation of at 24 bp upstream, and a single base A mutation at 7 bp upstream of 5' lateral exon 5. The other AA-TCC mutation was found at 40 bp upstream of 3' lateral exon 7, leading to terminator formation in codon of the *PTE*N gene and pre-termination of the open read frame with the PTEN protein product lacking of the C end that regulates the stability and activity of PTEN protein. Therefore, this mutation may play an important role in the occurrence and development of gastric cancer. The mutation in introns may have effects on the differentiated cut of PTEN transcription product due to the 3 point mutations in introns of the *PTE*N gene.

Furthermore, the study showed that PTEN protein expression in the 53 gastric cancer tissue samples was not significantly correlated with the clinicopathological parameters, such as gender and age of the patients, location and size of the carcinoma, distant metastasis, and embolization, but was significantly correlated with infiltrating depth, lymph node metastasis, and pTMN staging ($P < 0.05$). Along with the increasing infiltrating depth from level Ⅰ to level Ⅳ, the positive expression rate was gradually decreased from 92.9% to 54.2% ($P < 0.025$). There was a significant difference in lymph node metastasis ($P < 0.05$) between negative and positive PTEN expressions (88.9% *vs* 45.7%). The positive PTEN protein expression rate was significantly higher at pTMN stages Ⅰ and Ⅱ than at pTMN stages Ⅲ and Ⅳ ($P < 0.005$). These results suggest that PTEN may play an important role in regulation of infiltration and metastasis of gastric cancer cells. Abnormal expressions of PTEN may predict the metastasis and prognosis of gastric cancer[19-21]. Furthermore, positive PTEN protein rate was significantly higher in well-and moderately-differentiated gastric cancer than in poorly- differentiated gastric cancer ($P < 0.025$). There was also a significant difference in PTEN protein expression intensity among

Table 3 Correlation between PTEN protein expression and clinicopathological parameters in gastric cancer patients

Clinicopathology parameters	Case (*n*)	PTEN protein expression (*n*)		Positive rate (%)	*P*
		Negative	Positive		
Tissues					
Paracancerous	53	0	53	100.0	< 0.005
Gastric cancer	53	18	35	66.0	
Gender					
Male	41	15	26	63.4	
Female	12	3	9	66.7	
Age (yr)					
≤ 60	14	5	9	64.3	
> 60	39	13	26	66.7	
Size (cm)					
≤ 3	16	6	10	68.8	
> 3	37	12	25	64.9	
Location					
Antrum	30	10	20	66.7	
Gastric body and cardia	23	8	15	65.2	
Infiltrating depth					
T1, T2	14	1	13	92.9	< 0.025
T3	15	6	9	60.0	
T4	24	11	13	54.2	
Lymph node metastasis					
Without	21	2	19	90.5	< 0.01
With	32	16	16	50.0	
Distant metastasis					
Without	45	15	30	66.7	
With	8	3	5	62.5	
Embolization					
Without	8	1	7	87.5	
With	45	15	30	66.7	
Differentiation extent					
Moderate and high	26	4	22	84.6	< 0.025
Low	27	14	13	48.1	
PTNM staging					
Ⅰ, Ⅱ	19	1	18	94.7	< 0.005
Ⅲ, Ⅳ	34	17	17	50.0	

well, moderately and poorly differentiated gastric cancers ($P < 0.005$), suggesting that PTEN protein expression is significantly correlated with histological differentiation of gastric cancer. It is generally accepted that differentiation extent is an indicator for the prognosis of gastric cancer[22]. The gene is important in the process of inducing tumor differentiation and PTEN protein expression is of certain significance in the prognosis of gastric cancer patients.

In the present study, the mutation rate of gastric cancer was 9.4% (5/53), suggesting that except for gene mutations, other mechanisms are involved in the descending process of PTEN protein expression, such as over methylation of nucleotides C and G in promoter or enhancer. Abnormal methylation of CpG islands in promoter is considered one of the important mechanisms underlying gene deactivation and accumulation. The abnormal methylation is considered one of the main pathways promoting occurrence of gastric cancer because over methylation of tumor suppressor genes or other tumor-related genes, such as Rb, APC, p16, p15, hMLH1, E-cadherin, are found in malignant tumors. Another cause might be the abnormal regulation of PTEN protein decomposition pathways. Analysis of PTEN protein structure revealed that there were two homologous PESTs and one PSD-95/Dig/20-1 (PDZ) binding module at the C end of PTEN protein. Deletion or structure alteration in the region might result in PTEN protein prone to be decomposed. Because the total length of *PTEN* gene DNA is 218 bp including 9 exons and 8 introns, the exact mutation rate of the *PTEN* gene might be higher than 9.4%. The results of this study indicate that expression and mutation of the PTEN protein play an important role in the occurrence and development of gastric cancer.

It was reported that inactivation of PTEN induces infiltration and metastasis of tumors[23-28]. PTEN restrains attack and metastasis of tumor cells by regulating matrix metalloproteinases (MMPs) and vascular endothelial growth factor (VEGF)[29]. Abnormal expression of PTEN protein increases synthesis of MMPs and VEGF, thus leading to attack and metastasis of tumor cells. PTEN can also selectively increase dephosphorylation of focal adhesion kinase (FAK) to reduce cell transference by phosphated FAK[25,30-32]. Besides, PTEN protein and tensin have a homologous sequence[33]. Tensin is a cell matrix protein, which participates in adhesion to cells and extracellular matrix (ECM). Our study showed that PTEN could restrain cell transference as tensin.

In conclusion, abnormal expression of PTEN

protein is usually found in gastric cancer and related to tumor differentiation, infiltrating depth, lymph node metastasis and pTMN staging. PTEN may play an important role in the occurrence and development of gastric cancer. PTEN protein expression phenotype can be considered an indicator for the pathophysiological behavior of gastric cancer.

COMMENTS

Background

The occurrence and development of gastric cancer, like other malignant tumors, are a complicated process involving participation of polygene and many factors. It is generally considered that protein tyrosine phosphatase level plays an important role in the process. Mutation of the *PTEN* gene encoding for protein tyrosine phosphatase and abnormal expression of the PTEN protein are significantly correlated with the occurrence and development of malignant tumors, such as glioblastoma, prostate cancer, malignant melanoma, and breast cancer.

Research frontiers

Discovery of the *PTEN* gene is another important landmark in the field of anti-oncogenes. The relationship between *PTEN* gene and gastric carcinoma was analyzed for the genetic structure, expression and interaction with other genes in this study.

Innovations and breakthroughs

Few studies on *PTEN* gene mutation and protein expression in gastric cancer are available. However, cancer cells have not been isolated from normal cells that may lead to undetectable *PTEN* gene mutations because of plenty of normal genome DNAs.

Applications

PTEN protein phenotype can be used as an object index to judge the action of gastric carcinoma based on the cancer cells isolated from normal cells. In this study, we researched the *PTEN* gene mutations using PCR–SSCP–DNA sequencing technology, which can increase the detection rate of *PTEN* gene mutation, suggesting that it can be extended to research other tumor-related genes.

Peer review

This article describes mutation of the *PTEN* gene in patients with gastric carcinoma. The results indicate that *PTEN* gene plays an important role in the occurrence and development of gastric cancer.

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GASTRIC CANCER

Heparanase expression, degradation of basement membrane and low degree of infiltration by immunocytes correlate with invasion and progression of human gastric cancer

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Abstract

AIM: To disclose the mechanisms that accelerate or limit tumor invasion and metastasis in gastric cancer patients.

METHODS: The heparanase expression, continuity of basement, degree of infiltration by dendritic cells and lymphocytes in gastric cancer tissues from 33 the early and late stage patients were examined by immunohistochemistry, *in situ* hybridization and transmission electron microscopy.

RESULTS: Heparanase mRNA expression in the late stage patients with gastric cancer was stronger than that in the early stage gastric cancer patients. In the early stage gastric cancer tissues, basement membrane (BM) appeared intact, whereas in the late stage, discontinuous BM was often present. The density of S100 protein positive tumor infiltrating dendritic cells (TIDC) in the early stage gastric cancer tissues was higher than that in the late stage. The infiltrating degree of tumor infiltrating lymphocytes (TIL) in the early stage patients whose tumor tissues contained a high density of TIDC was significantly higher than that in the late stage gastric cancer tissues patients with a low density of TIDC. There were few cancer cells penetrated through the continuous BM of cancer nests in the early stage gastric cancers, but many cancer cells were found outside of the defective BM of cancer nests in the late stage.

CONCLUSION: Our results suggest that strong heparanase expression is related with the degradation of BM which allows or accelerates tumor invasion and metastasis. However, high density of TIDC and degree of infiltration by TIL are associated with tumor progression in human gastric cancers.

Key words: Heparanase; Basement membrane; Tumor infiltrating dendritic cell; Tumor infiltrating lymphocyte; Gastric cancer

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INTRODUCTION

Gastric cancer is one of the most aggressive malignant tumors, and its incidence is higher than that of any other gastrointestinal malignancy. The prognosis of patients with gastric cancer is often poor, due to tumor invasion and metastasis which are the most common causes of death in gastric cancer[1,2].

Degradation of basement membrane (BM) and extracellular matrix (ECM) around tumor is considered to be associated with invasion and metastasis of gastric cancer[3]. Heparanase is an endo-β-D-glucuronidase that specifically cleaves carbohydrate chain of heparan sulfate proteoglycans (HSPG)[4]. HSPGs are the main component of extracellular matrix and basement membrane which play a barrier to prevent tumor cells from invasion and metastasis[5]. Previous studies

have shown that heparanase, produced by malignant tumor cells, mediates degradation of heparan sulfate proteoglycans in the extracellular matrix and basement membrane around tumors, and their expression correlateds with the degree of tumor invasion and metastasis in several human malignant tumors[6-8].

Progression of malignant tumors is also restricted by host defense mechanisms[9,10]. The tumor infiltrating dendritic cells and lymphocytes are chief immunocytes that inhibite malignant tumor cells from invasion and metastasis. Many authors reported that the infiltration grade of tumor infiltrating dendritic cells are associated with patient survival and prognosis in a large variety of human malignancies[10-15]. Recent studies have shown that the number of tumor infiltrating lymphocytes is correlated with the progression of human carcinoma[16-18]. However, to our knowledge, the correlation between heparanase expression and infiltration degree of tumor infiltrating dendritic cells and lymphocytes has not been reported so far. The present study was, therefore, undertaken to clarify the relationships between heparanase mRNA expression, degree of degradation of basement membrane, density of tumor infiltrating dendritic cells, infiltrating degree of tumor infiltrating lymphocytes, and tumor invasion and progression in human gastric cancer patients.

MATERIALS AND METHODS

Tumor samples

Tissue samples were obtained from 33 patients with primary gastric cancer who underwent curative surgery in the Second Clinical Hospital of Harbin Medical University (Harbin, China). Ten patients had stage Ⅰ, 8 stage Ⅱ, 7 stage Ⅲ, 8 stage Ⅳ cancer according to the TNM classification (UICC, TNM classification, 5th Edition, 1997)[19]. Histological stage grouping was evaluated. Stages Ⅰ and Ⅱ (n = 18) were referred to the early stage, stages Ⅲ and Ⅳ (n = 15) were referred to the late stage. All fresh tumor tissues were divided into two parts, one part was fixed in 0.1 mol/L phosphate buffer (pH 7.4) containing 4% paraformaldehyde for immunohistochemistry and *in situ* hybridization, the other part was immersed in 0.1 mol/L phosphate buffer (pH 7.4) containing 2.5% glutaraldehyde for transmission electron microscopy.

In situ hybridization

Paraffin-embedded tissue sections were prepared for heparanase staining. Following reagents were purchased from Maxim Biotech (South San Francisco, CA, USA). Tissue sections (4 μm) were deparaffinized, dehydrated and incubated in 0.2 mol/L HCl for 20 min. After washed with 2 × SSC, the sections were incubated with proteinase K for 10 min at 37℃, fixed with PBS containing 4% paraformaldehyde for 5 min, washed with 2 × SSC, and then prehybridized for 2 h at 63℃ in a buffer containing 50% deionized formamide, 4 × SSC, 2 × Denhardt's solution and 250 μg/mL RNA. Hybridization was performed in 50% deionized formamide, 4 × SSC, 2 × Denhardt's solution, 10% dextran sulfate and 500 μg/mL RNA. The final concentration of DIG-labeled heparanase probe was about 500 ng/mL. The probe was placed on the section, covered with parafilm and incubated at 63℃ overnight in a moisture chamber. After hybridization, excess probes were removed by washing in 2 × SSC followed by RNase treatment with 100 U/mL RNase T1 at 37℃ for 30 min. The sections were washed at 65℃ in 2 × SSC for 10 min, washed three times in 0.2 × SSC and 50% deionized formamide (10 min each time), and incubated with an anti-DIG antibody conjugated with alkaline phosphatase. For the following color reaction, 5-bromo-4-chloro-3-indolyl phosphatase was used. Finally, the sections were counterstained with Mayer's hematoxylin.

Immunohistochemistry

Paraffin-embedded specimens were prepared for S100 protein immunohistochemical staining. The specimens were cut into 5-μm thick sections and mounted on glass slides. The sections were then deparaffinized in xylene for 20 min and dehydrated in ascending concentrations of ethanol. Endogenous peroxidase was blocked by incubating the sections with 3.0% H_2O_2 in methanol. After incubated in normal bovine serum for 10 min, the sections were incubated with anti-S100 protein antibody (Sigma, St. Louis, MO, USA) for 2 h at 37℃. After washed with PBS, the sections were incubated with biotinylated immunoglobulin and streptavidin conjugated to horseradish peroxidase (ABC kit, Sigma, St. Louis, MO, USA). Immunostaining was developed using DAB/ H_2O_2 solution. Finally, the sections were lightly counterstained with hematoxylin.

Transmission electron microscopy

Specimens fixed in 0.1 mol/L phosphate buffer (pH 7.4) containing 2.5% glutaraldehyde were rinsed with the phosphate buffer and postfixed in 0.1 mol/L phosphate buffer containing 1% OsO_4 for 2 h, dehydrated through ascending concentrations of ethanol, and embedded in Epon 812 using aclar film (Nisshin EM, Tokyo, Japan). Semi-thin sections were stained with toluidine blue and observed under a light microscope. Ultrathin sections were stained with uranyl acetate and lead citrate and examined under a H-600 transmission electron microscope (Hitachi, Tokyo, Japan).

Statistical analysis

Under the light microscope, S100 protein immunohistochemistry and heparanase mRNA stained sections were examined using the image analysis system computer software (Beihan Image Centre, Beijing, China). Twenty sections from each kind of staining were analyzed, two high-power fields (× 400) (each field is 0.255 mm^2) were randomly selected from each section. The number and area density of positive cells in each section were automatically calculated by the computer. The results were expressed as mean ± SD. Student's t-test was used to compare the S100 protein positive cells and heparanase expressing cells in the early stage gastric

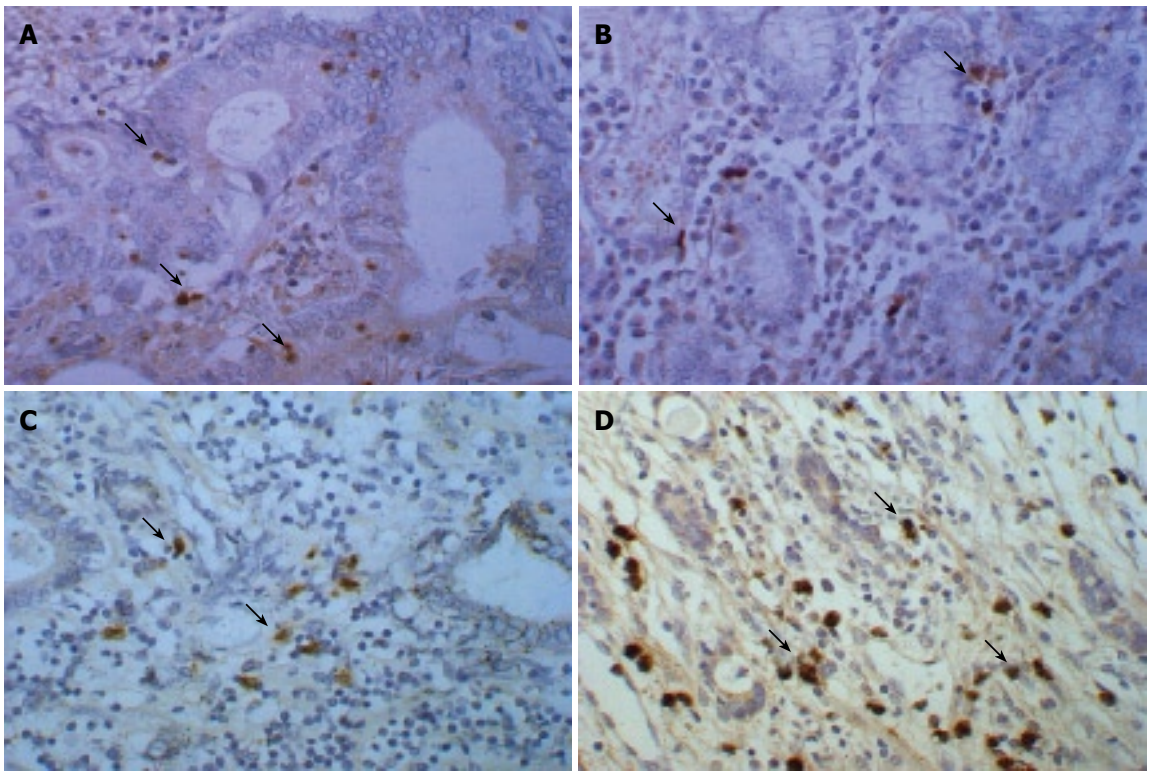

Figure 1 Expression and distribution of S100 protein and heparanase mRNA in gastric cancer tissues. Immunohistochemical staining of S100 protein (× 400) with a high density of tumor infiltrating dendritic cells positively stained for S100 protein in the early stage gastric cancer tissues (**A**) and a low density of such cells in the late stage gastric cancer tissues (**B**), heparanase mRNA expression by in situ hybridization (× 400) with a low heparanase mRNA expression level in the early stage gastric cancer tissues (**C**) and a high heparanase mRNA expression level in the late stage gastric cancer tissues (**D**) (Arrows: Positively expressed cells).

Table 1 Densities of S100 positive TIDC and heparanase expression in early and late gastric cancer tissues (mean ± SD)

	Densities of S100 positive TIDC			Heparanase expression		
	Early stage	Late stage	*P*-value	Early stage	Late stage	*P*-value
Cases (*n*)	18	15		18	15	
Number density	0.25 ± 0.19	0.03 ± 0.02	< 0.01	0.09 ± 0.08	0.33 ± 0.25	< 0.01
Area density	1.47 ± 1.15	0.21 ± 0.18	< 0.01	0.76 ± 0.64	3.47 ± 3.17	< 0.01

cancer tissues with those in the late stage gastric cancer tissues. $P < 0.05$ was considered statistically significant.

RESULTS

Distribution and density of tumor infiltrating dendritic cells in gastric cancer tissue

S100 protein positive cells showing typical morphology of dendritic cells and distinct cytoplasmic processes or veils, were detected in tissues from patients with gastric cancer at the early or late stage (Figure 1A and B). S100 protein positive cells were found mainly in stroma around the nests of cancer cells and connective tissue surrounding the tumor. In addition, S100 protein positive tumor infiltrating dendritic cells were also scattered among the cancer cells. Patients with gastric cancer at the early stage showed a high density of S100 protein positive tumor infiltrating dendritic cells (Figure 1A), while those at the late stage had a low density of S100 protein positive tumor infiltrating dendritic cells (Figure 1B). There was a significant difference in the density of S100 protein positive tumor infiltrating dendritic cells between gastric cancer patients at the early and late stage ($P < 0.01$, Table 1). The density of tumor infiltrating dendritic cells was significantly correlated with tumor invasion and clinical stage.

Heparanase mRNA expression in gastric cancer tissue

Heparanase mRNA positive labeling occurred mainly in cytoplasm and nuclei of gastric cancer cells. Heparanase mRNA was weakly expressed in the early stage gastric cancer tissues (Figure 1C) and strongly expressed in the late stage gastric cancer tissues (Figure 1D). The density of heparanase mRNA positive cells was significantly higher in the late stage gastric cancer tissues than in the early stage gastric cancer tissues ($P < 0.01$, Table 1). Heparanase mRNA expression was significantly correlated with invasion and TNM stage of gastric cancer.

Transmission electron microscopy (TEM)

The basement membrane was intact in the early stage

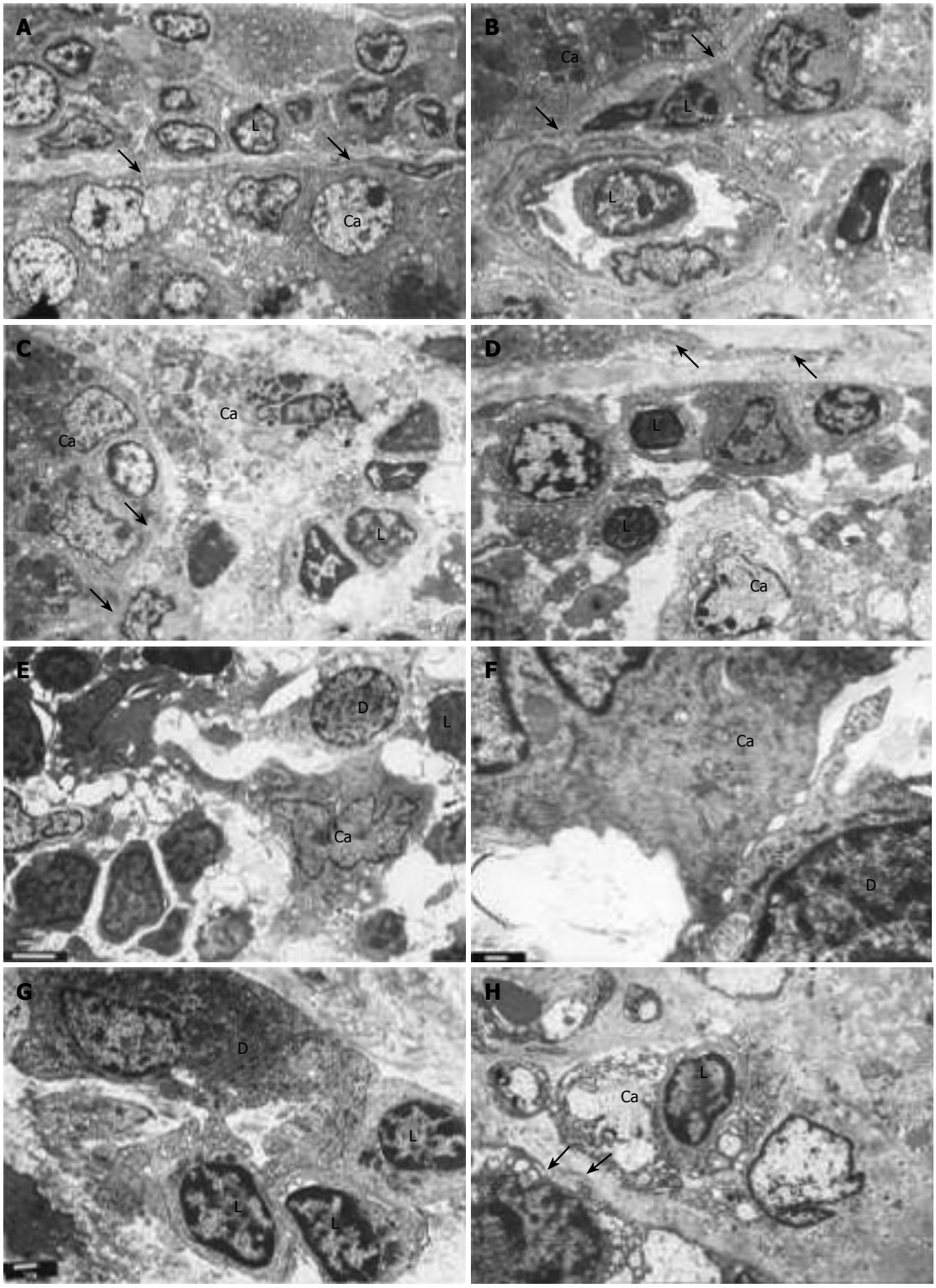

Figure 2 Transmission electron microscopy micrographs of the human gastric cancer tissues. (**A**)-(**D**) showing the early stage cancer tissues (Arrows indicate basement membrane). **A**: The continuous basement membrane which consisted of the electron-dense outer layer and the electron-lucent inner layer was observed. The numerous tumor infiltrating lymphocytes (L) were located in one side of the basement membrane (× 2500); **B** and **C**: The intact basement membrane was found on the margin of cancer nests. The tumor infiltrating lymphocytes (L) appeared around the cancer cell (Ca) (B × 4000; C × 3000); **D**: The cancer cell (Ca) was surrounded by the tumor infiltrating lymphocytes (L), and the basement membrane is clearly visualized (× 2500); (**E**)-(**H**) showing the late stage cancer tissues. **E**: The relationships were displayed between cancer cells (Ca) or tumor infiltrating lymphocytes (L) and tumor infiltrating dendritic cells (D). Note the tumor with absent basement membrane (× 4000); **F**: A higher magnification of **E** exhibited the contact relationship between the cancer cell (Ca) and tumor infiltrating dendritic cell (D) (× 20 000). **G**: The tumor infiltrating dendritic cell (D) was surrounded by several tumor infiltrating lymphocytes (L), and formed the dendritic cell-lymphocyte cluster (× 5000); **H**: The tumor infiltrating lymphocyte (L) appeared near the cancer cell (Ca), and the discontinuous or defective basement membrane of cancer nest can also be seen (double arrow) (× 5000).

gastric cancer tissue (Figure 2A-D). The continuous and well-formed basement membrane was found at the margin of

cancer nests in the early stage gastric cancer tissue (Figure 2B and C). The basement membrane was consisted of an electron-dense outer layer and an electron-lucent inner layer (Figure 2A and D). In contrast, the basement membrane at the rim of cancer nests was discontinuous and defective or absent in the late stage gastric cancer tissue (Figure 2E and H). Numerous tumor infiltrating lymphocytes were observed in the surrounding tissues of cancer nests and cells of the early stage gastric cancer (Figure 2B, C and D). In addition, many tumor infiltrating lymphocytes were arranged along one side of the basement membrane (Figure 2A). Lymphocytes in blood vessels were found near the cancer nests (Figure 2B), and invasion of cancer cells was noted outside of cancer nests (Figure 2C). The infiltrating degree of tumor infiltrating lymphocytes was high in the early stage gastric cancer tissues with intact basement membrane and few lymphocytes infiltrated into the tumor-surrounding tissue of the late stage gastric cancer with discontinuous basement membrane. Few cancer cells penetrated through the intact basement membrane of cancer nests in the early stage gastric cancer tissues, but many cancer cells were observed outside of the discontinuous basement membrane of cancer nests in the late stage gastric cancer tissues. There was a close contact between cancer cells or tumor infiltrating lymphocytes and tumor infiltrating dendritic cells (Figure 2E), and the contact between cancer cells and tumor infiltrating dendritic cells was also observed (Figure 2F). Many tumor infiltrating lymphocytes were distributed around the tumor infiltrating dendritic cells, forming a dendritic cell-lymphocyte cluster. Tumor infiltrating dendritic cells were closely contacted with tumor infiltrating lymphocytes (Figure 2G). Tumor infiltrating lymphocytes were also found near the cancer cells (Figure 2H).

DISCUSSION

It is generally accepted that one principal reason for the poor prognosis of patients with malignant tumors is the invasion and metastasis of cancer cells. The basement membrane plays an important role as a barrier in preventing cancer cells from invasion and metastasis[9]. The previous studies have demonstrated that heparanase which can degrade the basement membrane is one of the key enzymes involved in the tumor invasion and metastasis *in vivo*, such as pancreatic cancer, head and neck cancers, esophageal cancer, gastric cancer and colon cancer[7,8,20-22]. The heparanase can also shows *in vitro* human squamous cell carcinoma cell lines[23]. In the present study, we examined heparanase mRNA expression in the early and late stages of human gastric cancer by *in situ* hybridization. Our results show that heparanase mRNA expression was significantly higher in the late stage than in the early stage gastric cancer tissues ($P < 0.01$), and that heparanase mRNA expression was correlated significantly with tumor invasion and TNM stages of cancer.

Whether high expression of heparanase can promote the invasion of cancer cells by degrading the basement membrane remains unclear. Our TEM study has showed that intact basement membrane of cancer nests appeared in regions where heparanase expression was low in the early stage gastric cancer tissues, whereas discontinuous basement membrane of cancer nests was often present in places where heparanase expression was high in the late stage gastric cancer tissues. Our morphological observation directly proved that heparanase activity was associated with degradation of basement membrane. Lipponen[9] reported that invasion of superficial bladder cancer is related to the loss of continuous basement membrane, which is in agreement with our present TEM study.

In the present study, few cancer cells were observed to penetrate through the continuous basement membrane of cancer nests in the early stage gastric cancer tissue, but many cancer cells could be found outside of discontinuous basement membrane of cancer nests in the late stage gastric cancer tissue. These results suggest that the state of basement membrane, which is determined by heparanase mRNA levels, correlates with invasion of cancer cells. The present study also suggested that the discontinuity of basement membrane facilitate the invasion of cancer cells[23]. The discontinuity of basement membrane could probably results from the degradation of basement membrane by proteolytic enzymes, such as heparanase, which are presumably actively secreted by cancer cells[24].

The present study also showed that the state of basement membrane as a mechanical barrier and host immune defense system were interrelated. The immune defense system plays a critical role in preventing and limiting the development of malignant tumors[24]. The tumor infiltrating immunocytes situated around the tumor were considered a key factor for maintaining the status of local antitumor immunity[25]. The tumor infiltrating dendritic cells and lymphocytes are the main components of immunocytes in the tumor-surrounding tissues. Reportedly, the quantity of tumor infiltrating dendritic cells correlates with the clinical outcome of different tumor types[11,12,14,15]. Zeid and Muller demonstrated that the density of S100 positive dendritic cells in lung tumors is related to tumor subtype and differentiation, and a high dendritic cell density is associated with enhanced patient survival[11]. S100 protein has been widely used as a marker for identification of dendritic cells[26-28]. Tsujitani *et al* showed that the infiltration of dendritic cells is related to tumor invasion and lymph node metastasis in human gastric cancer[12]. A high number of dendritic cells in tumor tissue correlate with a good prognosis[29]. The present study showed that the density of S100 protein positive cells was higher in the early stage than in the late stage gastric cancer tissue ($P < 0.01$), suggesting that the density of tumor infiltrating dendritic cells correlates significantly with tumor invasion and clinical stages.

It was reported that invasion and metastasis of malignant tumor are related with the infiltrating degree of tumor infiltrating lymphocytes in tumor tissues[30]. Ropponen *et al* have shown the relationship between the number of tumor infiltrating lymphocytes and the prognosis of patients with colorectal cancer[31]. Aaltomaa *et al* demonstrated that a high number of

tumor infiltrating lymphocytes in tumor tissue correlate with a good prognosis of patients with breast cancer[32]. In the present study, the infiltrating degree of tumor infiltrating lymphocytes in the early stage gastric cancer patients whose tumor tissues contained a high density of tumor infiltrating dendritic cells was significantly higher than that in the late stage gastric cancer patients with a low density of these cells, indicating that the infiltrating degree of tumor infiltrating lymphocytes is associated with the progression of gastric cancer. These results suggest that there exists a certain relation between tumor infiltrating dendritic cells and lymphocytes. When the tumor becomes large, dendric cells and lymphocytes in the whole body are overwhelmed by a large number of tumor cells. In addition, these increased tumor cells will also prevent and limit infiltration by dendritic cells and lymphocytes. Thus, the decreases in tumor-infiltrating DCs and TILs may not be due to tumor development, but due to tumor growth.

Suzuki *et al* showed that dendritic cells are attached to groups of lymphocytes and form dendritic cell-lymphocyte clusters to promote T-cell activation for the generation of tumor-specific immunity in the invasive margin of the colorectal cancer stroma[33]. Dendritic cells present antigen to lymphocytes, stimulate naïve T lymphocyte proliferation and activation to kill tumor cells[34]. Bell *et al*[35] suggested that the peritumoral clustering of mature dendritic cells reflects a state in which they interact directly with clusters of tumor infiltrating lymphocytes to generate an antitumor immune response. In the present study, tumor infiltrating lymphocytes were distributed around tumor infiltrating dendritic cells and formed dendritic cell-lymphocyte cluster, and the close contacts were found between tumor infiltrating dendritic cell and tumor infiltrating lymphocytes. The contact between tumor infiltrating dendritic cells and lymphocytes may indicate the process that tumor infiltrating dendritic cells present antigen to lymphocytes to activate them for antitumor immunity[36,37].

Loss of integrity in basement membrane results from high heparanase expression. In the present study, few cancer cells were observed to penetrate through the continuous basement membrane of cancer nests in the early stage gastric cancer tissue, but many cancer cells could be found outside of the discontinuous basement membrane of cancer nests in the late stage gastric cancer tissue. These results suggest that the state of basement membrane, which is determined by heparanase, correlates with invasion of cancer cells. In the late stage gastric cancer tissue, increased cancer cells outside of discontinuous basement membrane of cancer nests could prevent and limit infiltration by dendritic cells and lymphocytes. Thus, heparanase expression or loss of integrity in basement membrane is associated with the infiltrating degree of tumor infiltrating dendritic cells and lymphocytes.

In summary, heparanase expression, degradation of basement membrane, density of tumor infiltrating dendritic cells and infiltrating degree of tumor infiltrating lymphocytes are associated with tumor invasion, TNM stages and progression in human gastric cancer. When the tumor has reached an advanced stage, discontinuous basement membrane, degraded by high expression heparanase, allows cancer cells to penetrate and is favorable to tumor invasion and metastasis, and can predict a poor prognosis of patients with gastric cancer. Moreover, at the late stage, a low degree of infiltration by dendritic cells and lymphocytes reflecting the presence of a weak local antitumor immune response in gastric cancer tissues also indicates that patients with less infiltrating immunocytes gastric cancer would have a poor prognosis, whereas the result is contrary in the early gastric cancer tissue. These factors including interactions between heparanase and basement membrane as well as between tumor infiltrating dendritic cells and lymphocytes may play a crucial role in tumor invasion and metastasis. Further study is required to understand the precise mechanism of interactions between both of them in the process of tumor invasion and metastasis.

COMMENTS

Background

The prognosis of patients with gastric cancer is often poor, due to tumor invasion and metastasis which are the most common causes for death of gastric cancer patients. It is crucially important to disclose the mechanisms underlying tumor invasion and metastasis. Heparanase, tumor infiltrating dendritic cells (TIDC) and tumor infiltrating lymphocytes (TIL) play a critical role in preventing and limiting the development of malignant tumors.

Research frontiers

Recent investigations have shown that heparanase expression which can degrade the basement membrane is one of the key enzymes involved in tumor invasion and metastasis, but few studies are available on the relationship between heparanase expression, basement membrane degradation, density of dendritic cells, infiltrating degree of lymphocytes, and tumor invasion and progression in gastric cancer patients.

Innovations and breakthroughs

High heparanase expression levels are related with the degradation of basement membrane which allows or accelerates tumor invasion and metastasis. However, high TIDC density and TIL infiltration degree are associated with progression of gastric cancer.

Applications

The study defined the mechanisms underlying tumor invasion and metastasis. Heparanase expression, basement membrane degradation, and TIDC and TIL infiltration degree, can be used as prognostic biomarkers for gastric cancer.

Terminology

Heparanase is an endo-β-D-glucuronidase that specifically cleaves the carbohydrate chain of heparan sulfate proteoglycan (HSPG). HSPG is the main component of extracellular matrix and basement membrane, which as a barrier can protect tumor cells from invasion and metastasis.

Peer review

This is a histopathological analysis of heparanase expression, dendric cells and lymphocytes infiltrating to tumor in patients gastric cancer at the early or late stage. In addition, the authors showed the electron microscopic pictures of gastric cancer tissue infiltrated by dendric cells and lymphocytes. This is an interesting report on heparanase expression in human gastric cancer.

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Killing of p53-deficient hepatoma cells by parvovirus H-1 and chemotherapeutics requires promyelocytic leukemia protein

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Abstract

AIM: To evaluate the synergistic targeting and killing of human hepatocellular carcinoma (HCC) cells lacking p53 by the oncolytic autonomous parvovirus (PV) H-1 and chemotherapeutic agents and its dependence on functional promyelocytic leukemia protein (PML).

METHODS: The role of p53 and PML in regulating cytotoxicity and gene transfer mediated by wild-type (wt) PV H-1 were explored in two pairs of isogenic human hepatoma cell lines with different p53 status. Furthermore, H-1 PV infection was combined with cytostatic drug treatment.

RESULTS: While the HCC cells with different p53 status studied were all susceptible to H-1 PV-induced apoptosis, the cytotoxicity of H-1 PV was more pronounced in p53-negative than in p53-positive cells. Apoptosis rates in p53-negative cell lines treated by genotoxic drugs were further enhanced by a treatment with H-1 PV. In flow cytometric analyses, H-1 PV infection resulted in a reduction of the mitochondrial transmembrane potential. In addition, H-1 PV cells showed a significant increase in PML expression. Knocking down PML expression resulted in a striking reduction of the level of H-1 PV infected tumor cell death.

CONCLUSION: H-1 PV is a suitable agent to circumvent the resistance of p53-negative HCC cells to genotoxic agents, and it enhances the apoptotic process which is dependent on functional PML. Thus, H-1 PV and its oncolytic vector derivatives may be considered as therapeutic options for HCC, particularly for p53-negative tumors.

Key words: Autonomous parvovirus; Apoptosis; p53; Promyelocytic leukemia protein; Human hepatocellular carcinoma; Hepatocytes

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INTRODUCTION

Abrogation of function of tumor suppressors such as p53 or the promyelocytic leukemia protein (PML) are common events in human tumors and lead to more aggressive cancer phenotypes[1,2]. At early stages during the process of carcinogenesis, activated oncogenes sensitize primary cells towards the p53-dependent stress response, in which the nuclear phosphoprotein p53 serves as a ge-

nomic stabilizer, inhibitor of cell cycle progression and angiogenesis, and facilitator of apoptosis[3-5]. In order to overcome this endogenous defense mechanism, cancer cell variants are strongly selected for p53 mutations, with p53 gene alterations identified in approximately half of all human tumors[6-8]. Thus, loss of p53 function usually results in a more aggressive cancer phenotype and worse clinical outcome. Studies using *in vitro* cell cultures and *in vivo* animal models demonstrated that the deficiency of p53 correlates with enhanced tumorigenesis, tumor-induced angiogenesis, and an increased resistance towards intracellular stresses which is mainly due to abrogation of an effective apoptotic response to chemotherapy or radiation[9].

The tumor suppressor PML is predominantly localized in distinct nuclear domains that are termed PML-nuclear bodies (PML-NBs), and consist of multiprotein complexes implicated in apoptosis regulation, cellular senescence, and antiviral response[10,11]. PML expression results in potent growth-suppressive[12] and apoptosis-inducing effects[13,14], and PML-deficient mice and cells exhibit defects in multiple apoptosis pathways[15]. One major goal of therapeutic oncology is to identify ways to kill the tumor cells that became resistant to conventional treatments due to their lack of functional p53 or PML[16]. The rapid expansion of the field of gene transfer technologies led to the development of retroviral or adenoviral p53 expression vectors and their use to restore sensitivity to genotoxic agents or to directly induce apoptosis in preclinical tumor models[17-20].

Promising new approaches to tumor-directed therapy include oncolytic parvoviruses (PVs), which are of particular interest, since they are endowed with oncolytic properties and also increase the host immune response by priming effector immune cells against the tumors[21-24]. The autonomous PV of the rat (H-1 PV) and its close relatives, such as the minute virus of mice (MVM) and in addition the most commonly used herpes simplex virus and adenovirus are emerging as promising candidates because of a number of their properties[25].

Notably, these viruses preferentially replicate in and kill transformed and tumor-derived cells in culture[21,22,26,27]. In addition, recombinant PVs have recently been produced with the aim to increase the anti-tumor effect of the natural viruses[28]. In particular, PVs may be suitable to target and kill tumor cells and simultaneously deliver appropriate transgenes, e.g. genes coding for immuno-stimulatory factors[25]. As H-1 PV is seldom pathogenic to its natural adult hosts[29] and infects humans without any apparent consequences[21,28], the prospects for the clinical use of PVs are intriguing. *In vivo*, these viruses may combat tumor development or repress established tumors, what makes them promising tools in cancer therapy[25].

The factors controlling the sensitivity of target (in particular human) cells to PV-induced killing are still largely unknown. Cells transformed with oncogenes display both an enhanced capacity for accumulating the viral cyotoxic nonstructural (NS) protein[22] and a greater intrinsic responsiveness to NS1-mediated killing[30]. On the other hand, our previous investigation of the cytotoxicity of H-1 PV in hepatoma cell cultures failed to go into a requirement for functional p53. So far, the inactivation of p53 was only found in human leukemia cells and transformed rat fibroblasts and correlates with a greater susceptibility to H-1 PV-induced cell killing[31].

Thus, in order to better understand the role of the cell genetic background and effector pathways in the H-1 PV-induced cytotoxicity, we compared two isogenic pairs of p53-positive *versus* negative human tumor cell lines of hepatocellular carcinoma (HCC) origin. This system allowed us to assess the impact of p53 on the susceptibility of host cells to both H-1 PV gene expression and killing activity, and H-1 PV vector-reduced reporter gene transduction. To further understand the molecular mechanism underlying H-1 PV-induced cell killing, another tumor suppressor, the PML, was investigated for its influence on H-1 PV infection. We used RNA interference to knock down PML expression in the described cellular systems, and determined the consequences for the outcome of H-1 PV infection with regard to the host cell p53 status.

The present study shows that H-1 PV triggers an apoptotic type of death in human HCC cells, and that p53 is dispensable for this process. In contrast, PML, which is induced by H-1 PV infection, helps PV killing carcinoma cells, irrespective of their p53 status. Given the known dependence of apoptosis induction by radiochemotherapeutic agents on the target cell p53 status[32], PVs appear to be suitable adjuvants to eliminate tumor cell populations resistant against these agents by means of combined treatments.

MATERIALS AND METHODS

Tumor cells

The Hep3B cells were derived from a HCC[33] and HepG2 cells from a human hepatoblastoma[32]. HepG2 cells were propagated in Dulbecco's modified Eagle medium (DMEM; Life Technologies GmbH, Karlsruhe, Germany), and Hep3B in Eagle minimal essential medium (Eurobio GmbH, Raunheim, Germany). Both media were supplemented with 10% fetal calf serum (FCS), 5 mol/L glutamine, 100 μg/mL penicillin, and 5 mol/L Hepes[21]. The Hep3B4P line is a Hep3B derivate stably transfected with tamoxifen-regulated wt p53-estrogen receptor chimera (p53-mERtm-pBabepuro)[34]. p53-mERtm-pBpuro contains the *Bam*HI fragment of human cDNA p53 cloned in frame with and N-terminally to a modified estrogen receptor containing a gly to arg mutation at residue 525[35]. This mutation renders the hormone binding domain insensitive to estradiol but responsive to the synthetic estrogen 4-OH-tamoxifen[35]. To induce p53 in the experiments, tamoxifen was added at a concentration of 750 nmol/L 1 d before H-1 PV infection.

HepG2 303 is a HepG2 derivative stably transfected with a dominant-negative p53 mutant (dn-p53) kindly provided by A. Levine (ΔV143A) as described by Schuler *et al*[36]. Since the dn-p53 transfection plasmid contained the puromycin resistance gene, HepG2 303 cells were

selected with puromycin (0.5 μg/mL) for 4 wk (4 consecutive days each week).

Virus infection

For infection, H-1 PV was produced in NB-E cells and purified over cesium chloride gradients as described earlier. Wild-type (wt) H-1 PV titration by plaque assays was performed according to published methods[26]. The multiplicity of infection (MOI) is given by the number of plaque-forming units (pfu) inoculated per cell. For experimental infections, exponentially growing cell cultures were incubated for 1 h with H-1 PV at indicated MOIs. Cells were cultured for up to 8 d post infection (p.i.).

Cell treatments

For combined treatment with H-1 PV and chemotherapeutics, cells were first infected with H-1 PV (MOI = 20 pfu/cell) in complete medium. One hour after infection, the chemotherapeutic agents Irinotecan (100 μg/mL), 5-Fluorouracil (5-FU) (5 μg/mL), or Cisplatin (0.25 μg/mL) were added, and cells were further incubated for 3 d at 37℃. Apoptosis rates were then quantitatively determined by FACS. Herein, the treated cells were harvested *via* trypisinization, washed with PBS, stained with propidium iodide and annexin V, and apoptosis levels were assessed, using the FACScan flow cytometry with CellQuest software (Becton Dickinson, San Jose, CA). Anticancer agents were purchased from Pfizer (Irinotecan), Hexal AG (5-FU), and Gry Pharma GmbH (Cisplatin).

Analysis of virus protein expression

Cultures were infected with H-1 PV at a MOI of 20 pfu/cell. After washing with PBS, cells were lysed in RIPA buffer (10 mol/L Tris-HCl, 150 mol/L NaCl, 1 mol/L EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, 5% SDS) containing protease inhibitors. Protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad, Munich, Germany). Total proteins (50 μg) were diluted into an equal volume, subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membrane (Amersham Pharmacia Biotech, Freiburg, Germany). Non-specific binding sites were blocked by incubating the membrane for 2 h in PBS containing 10% low-fat milk powder and 0.2% Tween-20 (Sigma, Deisenhofen, Germany). The blot was further incubated with the rabbit polyclonal antibody SP8 directed against carboxy-terminal peptides of NS1[37], then with an anti-rabbit peroxidase-conjugated antibody, and processed for enhanced chemoluminescence detection (Amersham Pharmacia Biotech, Freiburg, Germany).

Characterisation of the p53 and PML status of human tumor cells

Cultures grown for 48 h were processed for Western blotting as described above for viral proteins, p53 was detected using the monoclonal DO-7 antibody[38]. Actin was used as an internal loading control.

RNA interference

To knock down PML expression by RNA interference, the targeting oligonucleotide 5'-GAGCTCAAG TGCGACATCA-3' (*PML* sense) was inserted into the pSUPER vector. This target region is present in all PML isoforms and was verified by BLAST searches to confirm specificity. For control experiments, empty pSUPER vectors were used.

Measurement of PV-induced cell lysis

Hep3B and HepG2 cells were infected with H-1 PV at a MOI of 20 pfu/cell and further grown for 1 to 3 d. Cell permeabilization was then measured by using a standard toxicity assay (Toxilight, Cambrex Bio Science, Rockland Inc., USA) assessing the concentration of cellular adenylate kinase (AK) in culture supernatants according to the manufacturer's recommendations[39].

FACScan analysis of apoptosis

For quantification of the percentage of apoptotic cells in H-1 PV-infected cultures (MOI = 20 pfu/cell), adherent cells were dissociated with 0.25% trypsin and collected, together with cells floating in the medium, by centrifugation at 800 *g*. Cells were washed twice with PBS and stained with propidium iodide and annexin V (Becton Dickinson, Heidelberg, Germany). Fluorescence was measured with a minimum of 10 000 events per sample in a FACScan according to the manufacturer's instructions (Becton Dickinson). Data analysis was performed using the software Cell Quest (Becton Dickinson)[21].

Analysis of mitochondrial membrane potential

To measure the mitochondrial transmembrane potential, the cationic lipophilic fluorochrome JC-1 (5, 5, 6, 6-tetrachloro-1, 1, 3, 3-tetraethylbenzimidazolyl-carbocyanine iodide) (Molecular Probes, Inc., Eugene, OR) was used. JC-1 exists as a monomer in solution, emitting green fluorescence. In a reaction driven by the mitochondrial transmembrane potential, JC-1 can adopt a dimeric configuration and emit red fluorescence[40,41]. Mock- or H-1 PV-infected cultures (5×10^4 cells/mL) were incubated with JC-1 (5 μg/mL) for 20 min at room temperature in the dark, washed once in PBS and immediately analyzed by flow cytometry (FACScan, Becton Dickinson, Heidelberg, Germany) using Cellquest software. The red fluorescence of JC-1 indicates intact mitochondria, whereas green fluorescence shows monomeric JC-1 that remained unprocessed due to breakdown of the mitochondrial membrane potential[42]. After gating out small sized (i.e., non-cellular) debris, 10 000 events were collected for each analysis. The emitted green fluorescence signals were used as a measure for the loss of mitochondrial membrane potential[43].

Statistical analysis

Protein and (real time) gene expression values were analyzed for differences, using the one-sided Student's *t*-test. A *P*-value lower than 0.05 was considered as statistically significant.

RESULTS

Characterization of the p53 status in the hepatoma cell line pairs

In order to validate the system chosen to analyze the role of p53 and its effector pathways in H-1 PV-induced cellular cytotoxicity, we first confirmed the differential activity and expression of p53 in the two pairs of isogenic p53-positive and p53-negative HCC cell lines. The cell line HepG2 expresses functional wt p53 while Hep3B is a p53-null ($p53^{-/-}$) cell line[44]. Hep3B4P cells transfected with a tamoxifen-regulated p53-estrogen receptor chimera[34] were cultured with different concentrations of 4-OH-tamoxifen to induce p53. Upon transfection of a p53-transactivated luciferase construct (pConluc/pgupluc), 4-OH-tamoxifen induced dose-dependent the p53 activity, up to 25 times at 750 nmol/L of 4-OH-tamoxifen (data not shown). At this concentration, the induction of p53 expression was determined to have no detectable effects on the growth of non-infected cells in accordance to previous reports[38,45]. The cell lines studied differ in expression of p53 as expected (Figure 1A). HepG2 and HepG2 303 show p53 bands in both cases, because HepG2 303 is a HepG2 derivative stably transfected with a dominant-negative p53 mutant (dn-p53).

Production of parvoviral non-structural proteins in virus-infected p53 different tumor cells

In order to assess the effect of p53 on H-1 PV replication, expression of the replicative cytotoxic NS1 protein was analyzed in infected tumor cell line pairs. As illustrated in Figure 1B, all cultures were proficient in NS1 accumulation within a few days p.i., as detected by Western blot analysis. It was noteworthy; however the p53 expression somehow impaired the capacity for NS1 production, as apparent from the delayed appearance (HepG2 system) or reduced steady-state level (Hep3B4P system) of NS1 in the p53-positive cells. As previously reported[21], NS1 expression levels were in accordance with the respective amounts of viral DNA intermediates as well as luciferase activities of parvoviral vectors.

H-1 PV toxicity for human tumor cell pairs differing in their p53 status

We previously observed that a p53-negative hepatoma cell line was lightly susceptible to H-1 PV-induced cytotoxicity[21]. This prompted us to compare the p53-deficient cells with their p53-positive counterparts in terms of their relative sensitivities to H-1 PV-induced killing. The levels of cytotoxicity in H-1 PV-inoculated Hep3B4P and HepG2 cultures were first monitored by measuring cell permeabilization through the release of adenylate kinase (AK) in the medium for up to 3 d p.i. (Figure 2A). Compared to their mock-treated controls, increased cell death was observed for all hepatoma cell cultures.

In keeping with their above-mentioned greater efficiency in cytotoxic NS1 production, the p53-negative cell cultures moved to be significantly more sensitive to the toxic effect of H-1 PV than their p53-positive derivative. Indeed, supernatants from HepG2 303 cultures contained about twice the amounts of AK than those from the HepG2 parent after H-1 PV infection. Likewise, tamoxifen induction of p53 in the Hep3B4P cells correlated with an increase in their resistance to H-1 PV cytotoxicity.

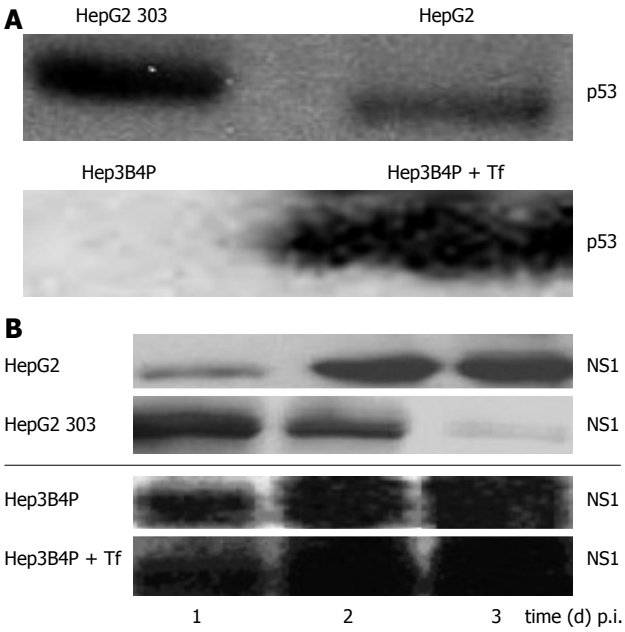

Figure 1 **A**: Characterisation of the p53 status and analysis of parvoviral proteins in different human tumor cells by Western blot. Cells were cultured for 2 d and lysed with RIPA buffer, and 50 μg of total protein was subjected to SDS-PAGE. For p53 protein detection, blots were incubated with the monoclonal DO-7 antibody; **B**: Production of parvoviral proteins in H-1 PV-infected p53 different tumor cells. Hep3B4P and HepG2 cells were H-1 PV-infected (MOI = 20 pfu/cell) and grown for 1 to 3 d. After lysis with RIPA buffer, 50 μg of total proteins were equally diluted and separated on SDS-PAGE. For parvoviral protein detection, blots were incubated with the NS1-specific antibody[37].

This difference was confirmed by FACS analysis quantifying the expression of annexin V, a known marker of apoptosis[30] (Figure 2B). In agreement with the above viability assay, H-1 PV infection induced annexin V in all hepatoma cells tested, yet to a higher level in p53-negative compared to p53-positive lines. Thus, p53 status correlated with sensitivity of hepatoma cells to the induction of H-1 PV-mediated apoptosis.

H-1 PV infection enhances depolarization of the inner mitochondrial membrane

The depolarization of the inner mitochondrial membrane has been associated with apoptosis in tumor cells exposed to different cytostatic agents or viruses[46]. This prompted us to investigate the effect of H-1 PV infection on this parameter in hepatoma cells. To this end, the profiles of JC-1 fluorescence were compared between mock- and H-1 PV-infected Hep3B4P cells. As illustrated in Figure 3, H-1 PV infection correlated with a striking increase in the fraction of hepatoma cells displaying depolarized mitochondrial membranes. In keeping with above data, this change occurred as soon as 1 d p.i. (Figure 3A) in p53-negative (Hep3B4P) in contrast to positive (Hep3B4P + Tf) cells. The p53-positive cells show an increase

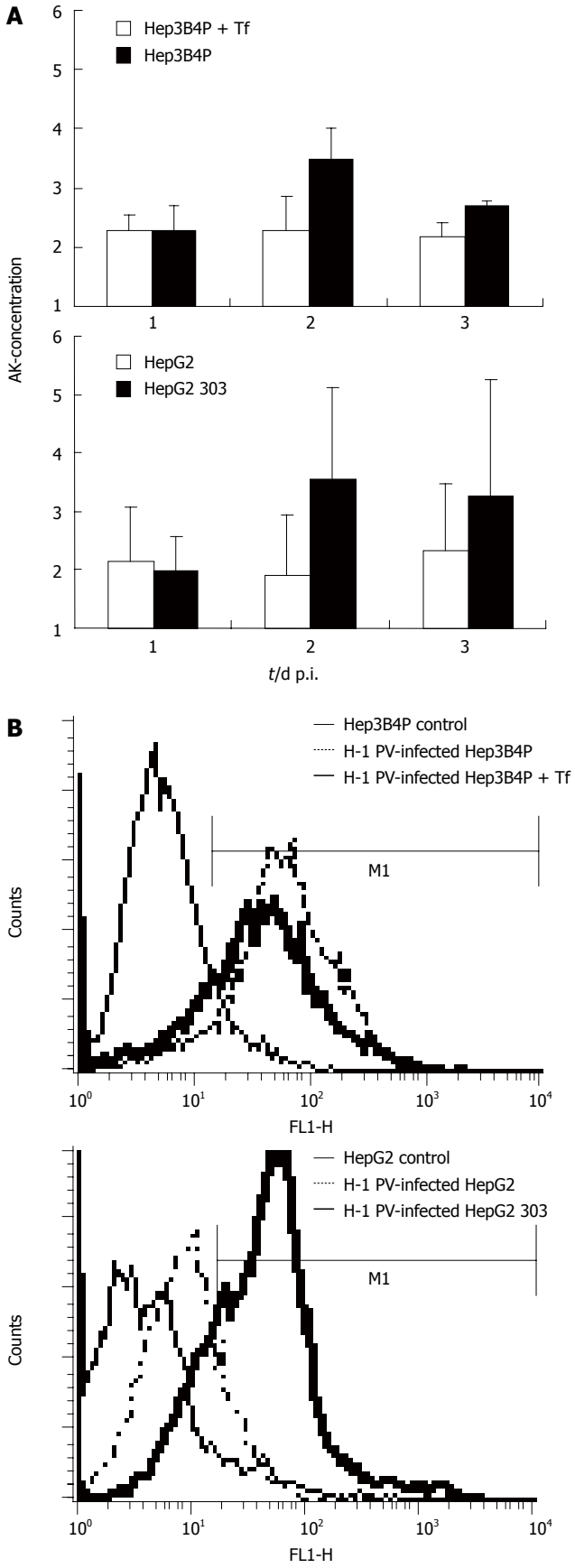

Figure 2 **A**: Cytotoxicity and induction of apoptosis in H-1 PV-infected p53 different tumor cell line pairs. The early damage of tumor cells upon H-1 PV infection was measured with a standardized toxicity test *via* supernatant adenylate kinase (AK) concentration; **B**: Induction of apoptosis in H-1 PV-infected tumor cells is shown in histograms for annexin V (FL1-H) of Hep3B4P and HepG2 cells. Data are given as mean values of triplicates.

of the depolarization of membran potential not before 2 d p.i. (Figure 3B).

Interplay of the tumor suppressor PML and H-1 PV infection

With the intention to identify factors influencing the sensitivity of hepatoma cells for H-1 PV, we determined whether the expression of the tumor suppressor protein PML was affected by H-1 PV-inoculation, and conversely, whether it had an impact on the outcome of infection. As shown in Figure 4, H-1 PV infection caused a strong increase in PML expression both on the protein (Figure 4A) and on the RNA (Figure 4B) level. To assess a possible role of PML in the control of H-1 PV-induced apoptosis in hepatoma cells, PML expression was reduced by RNA interference through expression of a short hairpin (sh) RNA (pSUPER-PML) which specifically targets PML. In contrast to the control pSUPER vector, the shPML construct strongly inhibited expression of endogenous PML in transfected HepG2 cells, irrespective of their p53 status (Figure 4B). Interestingly, knocking down of PML expression was found to correlate with a marked reduction of the efficiency of H-1 PV in inducing apoptosis in both HepG2 cells and their p53-negative HepG2 303 derivatives (Figure 4C). This result reveals PML to be centrally involved in regulation of apoptosis of hepatoma cells upon H-1 PV infection.

Treatment with chemotherapeutic agents combined with H-1 PV infection

The genetic drift of cancer cells leads to the appearance of variants resisting conventional genotoxic anticancer treatments. This prompted us to test whether hepatoma cells escaping chemotherapy may still be killed by H-1 PV, i.e. whether the combination of chemotherapeutics with H-1 PV meant an advantage. In a first step, this possibility was explored *in vitro* by determining whether H-1 PV infection enhanced the fraction of apoptotic hepatoma cells in cultures treated with cisplatin (Cis), irinotecan (Iri), or 5-FU. As illustrated in Figure 5B, in p53-deficient HepG2 303 cultures, H-1 PV was found to cooperate with all three agents in enhancing the overall fraction of treated cells undergoing apoptosis. Interestingly, the beneficial effect of the combined treatment or either of its individual components was not (cisplatin, irinotecan) or hardly (5-FU) significant in the p53-positive parental line HepG2 (Figure 5A). Therefore, p53 appeared to impair the cooperation of H-1 PV with genotoxic agents, possibly due to the fact that the chemotherapeutic agents enhanced the above-mentioned negative impact of p53 on the parvoviral life cycle. It is noteworthy that the p53-deficient HepG2 303 cells were less (irinotecan, 5-FU) or even more (cisplatin) sensitive to the chemotherapeutic tested, compared with the p53-positive HepG2 parent (Figure 5). This was surprising, given the usually lower susceptibility of p53-negative cells to the induction of apoptosis by genotoxic agents, but is not without precedent as p53 can be functionally replaced by re-

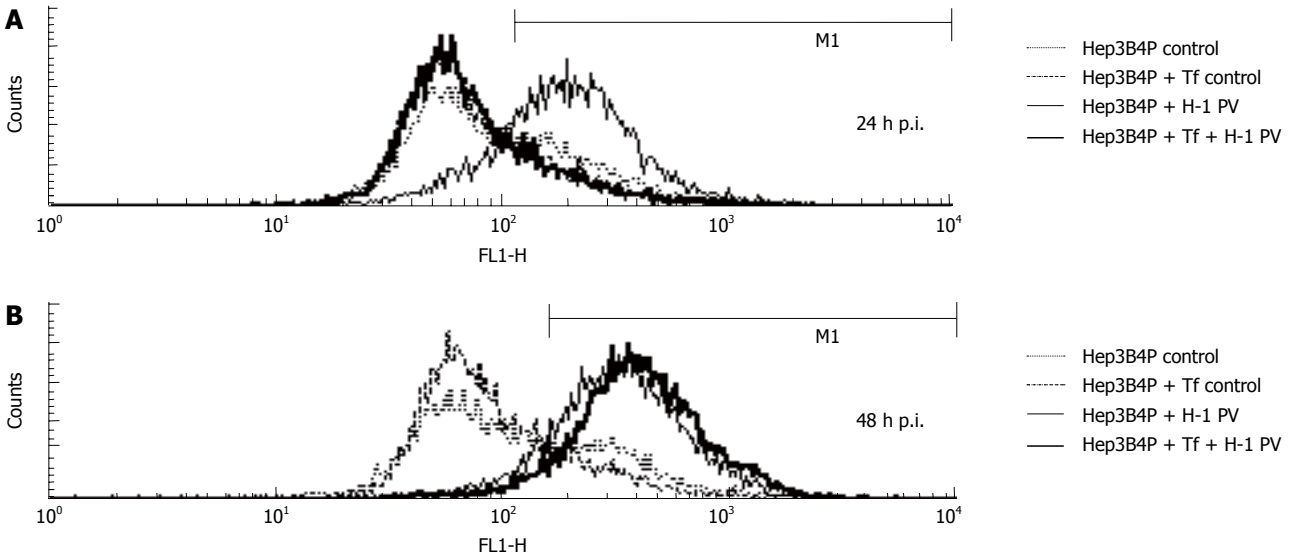

Figure 3 Analysis of mitochondrial membrane potential. Kinetics of reduction of the mitochondrial membrane potential. Hepatoma cells were left untreated (control) or infected (H-1 PV) for the indicated periods of time and analyzed by flow cytometry using the fluorochrome JC-1. The percentage of cells with decreased mitochondrial membrane potential is shown. After one (**A**) and two (**B**) days incubation, the percentage of cells with decreased mitochondrial membrane potential were determined by flow cytometry.

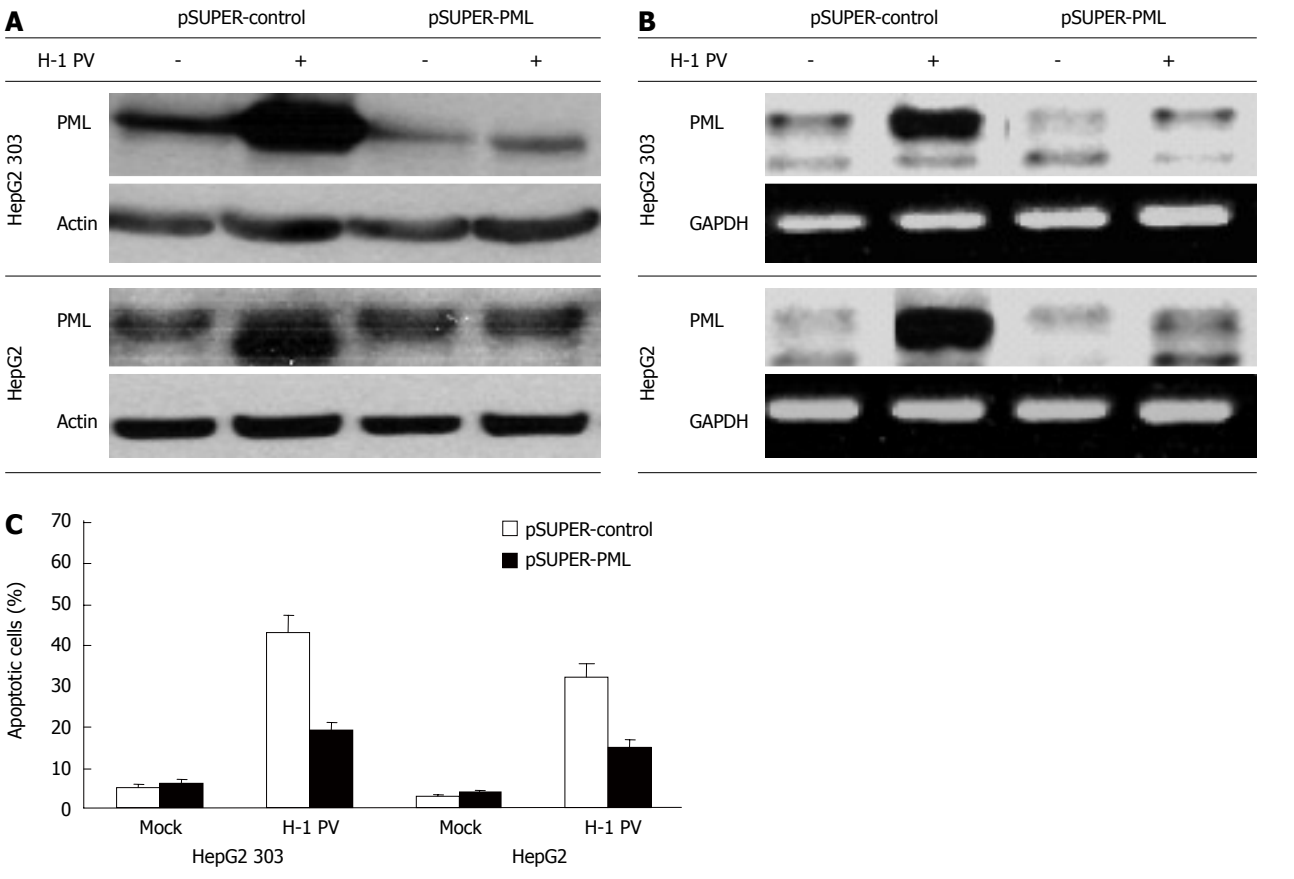

Figure 4 H-1 PV-induced apoptosis is mediated by PML. HepG2 ($p53^+$) and HepG2 303 ($p53^-$) cells were transfected with pSUPER or pSUPER-PML as indicated, and infected with H-1 PV for 48 h. Cells were harvested and subjected to Western blot (**A**) or PCR analysis (**B**). Hepatoma cells were treated as described, harvested, and subjected to cytotoxicity assay. Apoptosis was determined as described in Material and Methods (**C**).

lated products in drug-induced killing of some tumor cells[47].

DISCUSSION

The development of gene transfer techniques for tumor suppressor protein negative cancers is a rapidly expanding field: For HCC, different viruses have been assessed to specifically target p53-negative tumors or to transfer the wt p53 gene to reconstitute apoptotic pathways in tumor cells[17-20,44,48-51]. However, several of the viral delivery systems are limited by their immuno-

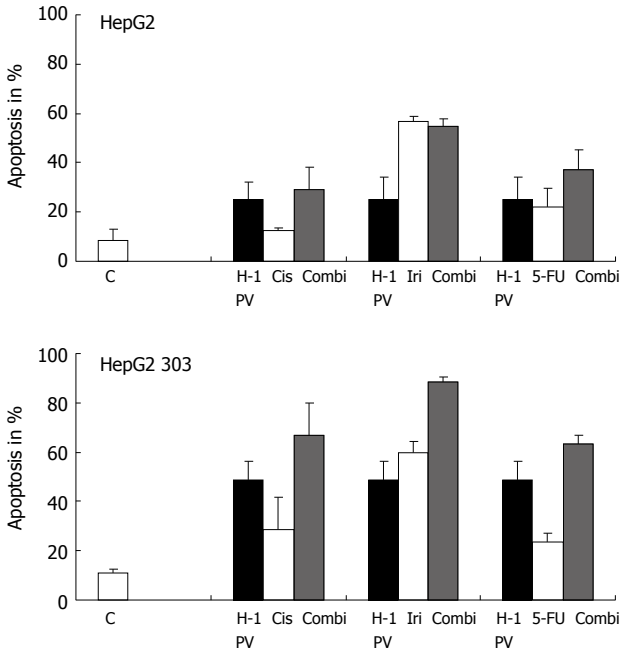

Figure 5 Treatment of p53 different tumor cells with chemotherapeutic agents. The p53 different HepG2 cells were treated with various chemotherapeutic agents alone or combined with H-1 PV infection (MOI = 20 pfu/cell). H-1 PV- or mock-infected cells were seeded into 6-well plates, and 1 h after infection cultures were treated with chemotherapeutic agents as indicated. Apoptosis was measured on day 3 by FACScan analysis. Data given represent mean values of triplicates.

genic or pathogenic side effects[52,53]. We have shown in this paper that oncolytic PV H-1 may be effective in the treatment of HCC. Even more, these oncolytic viruses directly targeting p53-negative carcinomas may be attractive alternative vectors as well as ideal tools for combination with classical chemotherapeutic agents. As the rat PV H-1 is seldom pathogenic to its natural adult hosts and infects humans without any apparent clinical consequences[28,29], we considered the PV H-1 for tumor cell-targeted therapy, in particular in p53-negative tumors. In addition, immune reaction to PVs, such as AAV or H-1 PV might not induce any severe side effects[21-24].

Thus, we first characterized the susceptibility to H-1 PV infection and cell killing of pairs of human tumor cells which differ in their wt p53 levels. In concordance with earlier data[21], H-1 PV-induced killing of tested tumor cells was dependent on MOI and correlated with NS1 expression levels. Similarly, amenability to gene transfer after recombinant PV infection was higher in p53 lacking cells[21]. Despite both isogenic HepG2 cells were susceptible to H-1 PV-induced apoptosis, cell death was more pronounced in HepG2 303. As well in other human and rodent cell systems studied so far, susceptibility to H-1 PV-induced cell killing correlated with the capacity of the host cells to sustain both, parvoviral DNA amplification and NS1 protein expression[20,28,54-56]. With this regard, earlier data revealed cellular processes underlying the PV-induced tumor cell killing. Further on p53 displayed a key role in the G_1/S checkpoint in response to DNA damage[57] as a regulator of cell cycle progression and a mediator of apoptosis in many cell lines[58]. Thus, p53 could prevent cell progression to S-Phase. According to this some S-phase factors such as p53 have been involved in the regulation of PV DNA replication[31,35]. Indeed, the rare H-1 PV-resistant variant clones named KS cells, isolated from the H-1 PV-susceptible human p53-negative erythroleukemia cell line K562, differed from the parental wt p53-positive cells by a reduced oncogenic potential in immunocompromised mice. Similarly, rat fibroblasts overexpressing mutant p53 protein were more sensitive to H-1 PV infection than parental cells[31].

Our data further demonstrate that H-1 PV induces significantly expression of PML on the RNA and protein level and, thus, increases the susceptibility to cell death in H-1 PV-infected tumor cells. The significance of this effect was clearly demonstrated by the knock down of PML in H-1 PV-infected cells with the consequence of impaired apoptosis upon H-1 PV infection. Recently, we showed that the hepatotropic hepatitis C virus (HCV) was able to impair apoptosis in hepatoma cells by inhibition of p53 function *via* interaction with PML[59]. Polypeptides from other viruses were also shown to interact with PML, and to disable its biological function in apoptosis regulation, growth suppression and cellular senescence. For example, adenoviral E1A protein abrogates oncogenic Ras- and PML-IV-induced cellular senescence by overriding PML function[60].

Furthermore PODs/PML bodies have been associated with transcription, cell growth, and antiviral responses[61,62]. DNA and RNA viruses also frequently target PODs, presumably to facilitate the early stages of transcription and replication[63-67]. PODs can also be targeted for reorganization following viral infection[66]. For example, adenovirus protein E4-ORF3 localizes to PODs/PML bodies, thereby causing a physical restructuring of the bodies from spherical to extended fibril-like structures termed nuclear tracks[66]. Additionally, many viruses induce interferon expression, what increases the size and number of PODs[62,68]. Although PV infection did not induce an interferon response, a dramatic relocalization of PODs has been seen late in MVM infection. However, Czipluch *et al*[69] suggested that H-1 virus does not target known nuclear bodies for DNA replication but rather induced the formation of a novel structure in the nucleus of infected cells. Within that study, PML-expression was not directly investigated.

In addition, PVs were recently reported to replicate in association with distinct nuclear bodies[69], which appear to lately merge with PML and PML-NBs[70]. PML may interact with viral products and participate in the regulation of virus replication. PML proteins may thus modulate viral cytopathic effects, in keeping with the above-mentioned involvement of PML in growth inhibition and death processes. As PV H-1 infection enhances PML expression and function, this might be a central molecular mechanism for an effective treatment of HCC.

We furthermore examined the effects of various chemotherapeutic agents in combination with H-1 PV

infection on growth inhibition using isogenic cancer cells with different p53 status. H-1 PV infection enhanced the cytotoxicity of chemotherapeutic agents in the treatment of two HCC. In p53-deficient HepG2 303 cultures, H-1 PV was found to cooperate with all three agents in enhancing the overall fraction of treated cells undergoing apoptosis. The treatment with Irinotecan, 5-Fluorouracil and Cisplatin combined with H-1 PV infection more strongly inhibited the growth of the p53-negative HepG2 303 cells than treatment with chemotherapeutics alone. Therefore H-1 PV infection enhances cytostatic drug therapy in p53-negative tumors. Furthermore, irrespective of the p53 status, H-1 PV was able to induce programmed cell death in these human tumor cells, as it was also shown for other PVs[71]. Chemotherapeutic treatment alone did not induce such a high apoptosis rate compared to combined treatment with H-1 PV. Comparable data have also been published for other oncolytic viruses[72-75]. Thus, our data show a beneficial interaction between chemotherapy and oncolytic viral therapy and suggest that H-1 PV infection may enhance the effectiveness of chemotherapeutic agents in the treatment of HCC.

In summary, our results strongly suggest that p53-impaired tumors-which have a poor prognosis-may be particularly suitable to PV H-1-induced therapy[39,43,45]. Though p53 deficiency in tumors may induce resistance to chemotherapeutic agents, this will not affect the tumor cell susceptibility to H-1 PV-induced oncolytic infections[51]. As recombinant H-1 PV had a high capacity to transduce transgenes in these cells, the therapeutic potential of H-1 PV-based recombinant vectors carrying suicide genes or cytokines should be further assessed in p53-negative tumors. The PV H-1 may then also overcome other tumor resistance mechanisms against autocrine and paracrine apoptotic triggers developed in these tumor entities[50,51]. We conclude that our strategy using H-1 PV infection in combination with chemotherapeutic treatment can enhance the cytotoxic effect of anti-cancer agents. Furthermore, H-1 PV induced the expression of PML, thus increased the susceptibility to cell death in H-1 PV-infected tumor cells. So PML may operate as a positive element which controls in a direct or indirect way the susceptibility of hepatoma cells to apoptosis-activity of H-1 PV.

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COMMENTS

Background

Oncolytic parvoviruses (PVs) are endowed with oncolytic properties and also increase the host immune response against the tumor by priming effector cells.

Research frontiers

Authors evaluated the synergistic targeting and killing of human hepatocellular carcinoma (HCC) cells lacking p53 by PV H-1 and chemotherapeutic agents.

Innovations and breakthroughs

Analysing the regulating the cell killing pathways and gene transfer mediated by PV H-1 in pairs of human hepatocellular cell lines with different p53 status, H-1 PV is quite a suitable agent to circumvent the resistance of p53-negative HCC to genotoxic agents, and enhances the apoptotic process which is dependent on functional PML.

Applications

Especially for p53-negative human tumors authors consider PV H-1 as therapeutic option for human HCC.

Peer review

This manuscript described that H-1 PV is a novel agent for treating p53 negative HCC *via* the induction of PML and apoptosis. This study is a well designed, well exerted, and well written manuscript.

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Bcl-x_L and Myeloid cell leukaemia-1 contribute to apoptosis resistance of colorectal cancer cells

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Abstract

AIM: To explore the role of Bcl-x_L and Myeloid cell leukaemia (Mcl)-1 for the apoptosis resistance of colorectal carcinoma (CRC) cells towards current treatment modalities.

METHODS: Bcl-x_L and Mcl-1 mRNA and protein expression were analyzed in CRC cell lines as well as human CRC tissue by Western blot, quantitative PCR and immunohistochemistry. Bcl-x_L and Mcl-1 protein expression was knocked down or increased in CRC cell lines by applying specific siRNAs or expression plasmids, respectively. After modulation of protein expression, CRC cells were treated with chemotherapeutic agents, an antagonistic epidermal growth factor receptor (EGFR1) antibody, an EGFR1 tyrosine kinase inhibitor, or with the death receptor ligand TRAIL. Apoptosis induction and cell viability were analyzed.

RESULTS: Here we show that in human CRC tissue and various CRC cell lines both Bcl-x_L and Mcl-1 are expressed. Bcl-x_L expression was higher in CRC tissue than in surrounding non-malignant tissue, both on protein and mRNA level. *Mcl-1* mRNA expression was significantly lower in malignant tissues. However, protein expression was slightly higher. Viability rates of CRC cells were significantly decreased after knock down of Bcl-x_L expression, and, to a lower extent, after knock down of Mcl-1 expression. Furthermore, cells with reduced Bcl-x_L or Mcl-1 expression was more sensitive towards oxaliplatin- and irinotecan-induced apoptosis, and in the case of Bcl-x_L also towards 5-FU-induced apoptosis. On the other hand, upregulation of Bcl-x_L by transfection of an expression plasmid decreased chemotherapeutic drug-induced apoptosis. EGF treatment clearly induced Bcl-x_L and Mcl-1 expression in CRC cells. Apoptosis induction upon EGFR1 blockage by cetuximab or PD168393 was increased by inhibiting Mcl-1 and Bcl-x_L expression. More strikingly, CD95- and TRAIL-induced apoptosis was increased by Bcl-x_L knock down.

CONCLUSION: Our data suggest that Bcl-x_L and, to a lower extent, Mcl-1, are important anti-apoptotic factors in CRC. Specific downregulation of Bcl-x_L is a promising approach to sensitize CRC cells towards chemotherapy and targeted therapy.

Key words: Colorectal carcinoma; Bcl-x_L; Myeloid cell leukaemia-1; Epidermal growth factor receptor 1; Apoptosis; 5-fluorouracil; Irinotecan; Oxaliplatin

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INTRODUCTION

Colorectal carcinoma (CRC) is one of the most common malignancies in the Western world. In palliative care, novel treatment approaches including combination of chemotherapy and targeted therapies, such as epidermal growth factor receptor (EGFR) 1 blockage, have improved survival of cancer patients[1]. However, 5-year survival of patients with metastatic CRC remains < 5%. Current established systemic therapy options include 5-fluorouracil (5-FU), oxaliplatin, irinotecan, the EGFR1 antibody cetuximab and the vascular endothelial growth factor (VEGF)-A antibody bevacizumab.

Apoptosis is a genetically programmed process of controlled suicide, which is critical for multicellular organisms during development and for tissue homeostasis. However, in cancer, tumor cells acquire resistance to apoptosis. Thus, the ratio of apoptosis and cell division is altered, resulting in a net gain of malignant tissue. Additionally, defects in apoptosis signalling in cancer cells impair response to therapy and contribute to the limited efficacy of different therapy regimens in metastatic disease[2].

Stabilization of mitochondrial integrity is a key mechanism for the survival of a malignant cell and its resistance to therapy[3]. Mitochondrial integrity is regulated by pro- and anti-apoptotic members of the Bcl-2 family, such as Bcl-x_L and Mcl-1 (Myeloid cell leukaemia-1, anti-apoptotic) and Bid, Bad and Bax (pro-apoptotic). Mcl-1 is essential for development, differentiation and survival in a variety of cell types[4,5]. It is involved in important interactions of Bcl-2 family members and thereby regulates mitochondrial activation[6]. Mcl-1 protein levels are elevated in various human tumors, such as hepatocellular carcinoma[7] and non-small cell lung cancer[8]. Importantly, it contributes to the resistance of cancer cells towards apoptosis induction[6,9]. Downregulation of Mcl-1 has been shown to sensitize cancer cells towards apoptosis induction, e.g. after treatment with the death receptor ligand TRAIL [tumor necrosis factor (TNF)-related apoptosis-inducing ligand][10,11]. In addition, Mcl-1 degradation is necessary for mitochondrial activation after genotoxic stress[12]. Like Mcl-1, Bcl-x_L is known to promote cell survival by counteracting pro-apoptotic Bcl-2 family members, such as Bim, Bax and Bid. In cancer, overexpression of Bcl-x_L is associated with tumor progression, poor prognosis and resistance to chemotherapy. In CRC, Bcl-x_L expression is correlated with an advanced disease stage[13]. A role of Bcl-x_L in cancer was first suggested when it was found that expression of Her-2/Neu in breast cancer cells increased Bcl-x_L expression and rendered cells resistant to tamoxifen-induced apoptosis[14]. Ectopic expression of Bcl-x_L in CRC blocks curcumin-induced apoptosis[15]. On the other hand, downregulation of Bcl-x_L by antisense technique induces cell death, e.g. after treatment with chemotherapeutic drugs[16,17]. An important trigger for Bcl-x_L expression in CRC is NF-κB[18]. CRC cells frequently harbor genetic aberrations that promote NF-κB-mediated induction of Bcl-x_L.

The last decade has ushered in new advances for the treatment of patients with CRC. The older cytotoxic chemotherapy drug 5-FU underwent new formulation, and two new drugs, oxaliplatin and irinotecan, were investigated as adjunctive therapies. Finally, targeted therapies, including monoclonal antibodies against VEGF-A (bevacizumab) and EGFR1 (cetuximab), are now standard treatment for metastatic CRC. For patients with metastatic disease, the survival rate has doubled. Among others, a promising approach to overcome apoptosis resistance of CRC cells is the engagement of the death receptors belonging to the tumor necrosis factor receptor gene superfamily with the death ligand TRAIL (Apo2L)[19].

In our study, we investigated the role of the anti-apoptotic Bcl-2 family members Bcl-x_L and Mcl-1 for the apoptosis sensitivity of CRC. Both Bcl-2 family proteins were specifically modulated in CRC cells by RNA interference and overexpression, respectively, and the impact on apoptosis sensitivity towards chemotherapy and targeted therapy including TRAIL and EGFR1 blockage was explored.

MATERIALS AND METHODS

Reagents and cell lines

SW480, HT29, Caco-2 (all isolated from primary tumor tissue) and SW620 (derived from lymph node metastasis), all human CRC cell lines (adenocarcinomas), were purchased from ATCC. Cell lines were cultured in RPMI 1640 (Invitrogen, Karlsruhe, Germany), supplemented with 10% fetal calf serum (FCS, Biochrom, Berlin, Germany), Pen/Strep (1%) (PAA Laboratories, Pasching, Austria), HEPES (1%) (Cambrex, Verviers, Belgium) and L-Glutamin (1%) (Cambrex). Cells were cultivated in reduced medium (FCS concentration decreased to 0.5%) in all experiments. Reagents were purchased from the following suppliers: chemotherapeutic agents from Sigma (Deisenhofen, Germany); TRAIL (with enhancer, applied in a concentration of 1 ng/mL) from Alexis Biochemicals (San Diego, CA, USA); PD168393 from Calbiochem (Schwalbach, Germany); Protein A (for co-treatment with anti-APO-1, in a concentration of 10 ng/mL) and EGF

from Sigma. Cetuximab was supplied by Merck Pharma (Darmstadt, Germany). Anti-APO-1 was kindly provided by Peter H. Krammer (German Cancer Research Center).

Tissue samples

CRC tissue samples as well as non-neoplastic colorectal tissues were obtained from patients undergoing elective surgery for colorectal cancer at the University of Mainz. Analysis of CRC samples was approved by the local ethics committee. The morphological classification of the carcinomas was conducted according to WHO specifications. Tissues samples were used for immunhistochemical staining as well as for mRNA extraction.

Immunohistochemical staining

Paraffin-embedded tissue sections (which all included carcinoma as well as normal epithelial cells in one section) were subjected to immunostaining, using a biotin/streptavidin-peroxidase technique (Vector Laboratories Inc., Burlingame, CA). They were deparaffinized in xylene and dehydrated in ethanol, and dried in a steamer with 10 mmol Na-citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked by incubating the slices for 5 min with 3.0% hydrogen peroxide at room temperature, followed by washing in TPBS (0.5% TWEEN in PBS). The sections were then incubated for 30 min at room temperature with TNB (1% BSA, 0.5% protein-blocking reagent in TBS) prior to an overnight incubation at 4℃ with polyclonal rabbit Bcl-xS/$_L$ (clone S-18, Santa Cruz Biotechnology Inc., Santa Cruz, California) or polyclonal rabbit Mcl-1 (S-19, Santa Cruz). Both were diluted 1:160 in TNB. Bound antibody was detected using biotinylated anti-rabbit IgG secondary antibody (Vector) and streptavidin-peroxidase complex (Vector), followed by incubating with diaminobenzidine as substrate. Sections were counterstained with Mayer's haematoxylin. As negative controls, sections were incubated in the presence of nonimmunized rabbit IgG as first antibody.

Viability test

Cell viability was determined by a colorimetric 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. CRC cell lines were seeded onto 12-well plates. On day 1 after seeding, cells were treated as indicated. 100 μL MTT (5 mg/mL) was added to each well. After 4 h incubation at 37℃ the supernatant was discarded and cells were washed with PBS. For cell lysis 0.5 mL 1-propanol was added for another 20 min. After transfer of 100 μL of each sample to a flat-bottomed 96-well microtiter plate; the optical density was determined at 550 nm. The viability of "1" was defined as the absorbance obtained from mock transfected cells or untreated cells, respectively.

Detection of apoptosis

CRC cell lines were seeded onto 12-well plates. On day 1 after seeding, cells were treated as indicated. Cells were then collected, washed, and resuspended in lysis buffer containing 0.1% (w/v) sodium citrate, 0.1% (v/v) Triton X-100 and 50 μg/mL propidium iodide (Sigma). After overnight incubation at 4℃, nuclei from apoptotic cells were quantified by flow cytometry according to the method of Nicoletti *et al*[20].

Cell lysis and Western blotting

Cell lysis and Western blotting were performed as described before[7]. Immunodetection was performed using the indicated primary antibodies: anti-Mcl-1 (S19) (Santa Cruz Biotechnology, Heidelberg, Germany), anti-Bcl-x_L (Labvision/NeoMarkers, Warm Springs Blvd. Fremont, Canada), and mouse anti-α-Tubulin clone B-5-1-2 (Sigma).

RNAi and transfection

For small interfering RNA (siRNA)-mediated knock down of *Mcl-1* and *Bcl-x$_L$*, the following siRNA sequences were applied (MWG Biotech, Ebersberg, Germany): *Mcl-1*, 5'-aaguaucacagacguucucTT-3' (sense) and 5'-gagaacgucugugauacuuTT-3' (antisense). *Bcl-x$_L$*, 5'-gcu uggauaaagaugcaaTT-3' (sense) and 5'-uugcaucuuuau cccaagcAG-3' (antisense). As a non-silencing control, siRNA specific for green fluorescent protein (*GFP*) was used: 5'-ggcuacguccaggagcgcaccTT-3' (sense) and 5'-ggu gcgcuccuggacguagccTT-3' (antisense), where capitals represent deoxyribonucleotides and lower case letters represent ribonucleotides. SW480 cells were transiently transfected with Lipofectamin RNAiMAX (Invitrogen, Karlsruhe, Germany) according to the manufacturer's protocol and analyzed 24 h after transfection. For Mcl-1 and Bcl-x_L expression, we applied specific expression vectors (pEF4$_{Mcl-1}$ or pcDNA3$_{Bcl-xL}$) or the corresponding empty vectors (pEF4$_{empty}$ or pcDNA3$_{empty}$, respectively), all kindly provided by Peter H. Krammer, German Cancer Research Center (Heidelberg, Germany). SW480 cells were transfected with plasmids using Transfectin (Biorad, München, Germany) according to the manufacturer's protocol.

Real-time quantitative polymerase chain reaction (RT-QPCR)

To analyze RNAi efficiency, total RNA from CRC cells was extracted using RNeasy Mini Kit (Qiagen) 24 h after transfection of siRNA. One μg of total RNA was reverse transcribed using an oligo-dT primer with the Omniscript RT kit (Qiagen) and afterwards analyzed for specific mRNA expression by RT-QPCR using the QuantiTect SYBR Green PCR Kit (Qiagen) and the following primers: *Actin* forward: 5'-GGACTTCGAGCAAGAGATGG-3', *Actin* reverse: 5'-AGCACTGTGTTGGCGTACAG-3', *Mcl-1* forward: 5'-TAAGGACAAAACGGGACTGG-3', *Mcl-1* reverse: 5'-ACCAGCTCCTACTCCAGCAA-3'. *Bcl-x$_L$* forward: 5'-GTAAACTGGGGTCGCATTGT-3', *Bcl-x$_L$* reverse: 5'-TGCTGCATTGTTCCCATAGA-3'. The relative increase in reporter fluorescent dye emission was monitored. The level of *Mcl-1* or *Bcl-x$_L$* (gene of interest, *GOI*) mRNA, respectively, relative to actin, was calculated using the formula: Relative *GOI* mRNA expression = 2 [C_t ($GOI_{control}$) - C_t ($GOI_{treated}$) + C_t ($Actin_{treated}$) - C_t ($Actin_{control}$)], where C_t

is defined as the number of the cycle in which emission exceeds an arbitrarily defined threshold. For evaluation of *Bcl-x$_L$* and *Mcl-1* mRNA expression in tumor as well as non-neoplastic colon tissues, RP II instead of actin was measured as housekeeping gene: *RP II* forward: 5'-GCACCACGTCCAATGACAT-3', *RP II* reverse: 5'-GTGCGGCTGCTTCCATAA-3'.

Statistical analysis

All results are expressed as mean ± SD. Data were analyzed by Student's *t*-test (paired, two sided). $P < 0.05$ was considered significant.

RESULTS

Expression of the anti-apoptotic Bcl-2 family members Bcl-x$_L$ and Mcl-1 in CRC

Apoptosis resistance is a well-known phenomenon which counteracts chemotherapeutic drug-induced cell death of CRC cells. Anti-apoptotic Bcl-2 family members such as Bcl-x$_L$ and Mcl-1 contribute to the apoptosis resistance in different tumor entities. First, we analyzed expression of Bcl-x$_L$ and Mcl-1 in various CRC cell lines. All cell lines tested showed a profound expression of Mcl-1 on protein level (Figure 1A). Bcl-x$_L$ expression was rather low in HT29 and Caco-2 and high in SW480 cells (Figure 1A).

Next, we analyzed expression of *Bcl-x$_L$* and *Mcl-1* mRNA in human CRC tissues by quantitative PCR. Bcl-x$_L$ levels were higher in CRC tissues compared to non-malignant, adjacent tissue (Median of relative expression: 1.2, $n = 9$, $P < 0.2$, not significant, Figure 1B). Six of 9 patients showed a higher *Bcl-x$_L$* expression, 2 patients showed a lower expression, and in 1 patient, expression was virtually equal. *Mcl-1* mRNA expression was significantly lower in carcinoma tissue compared to non-malignant tissue (Median of relative expression: 0.41, $n = 9$, $P < 0.01$). In addition, we performed immunohistochemical analysis of Bcl-x$_L$ and Mcl-1 in CRC. In all tissues tested ($n = 6$), expression of Bcl-x$_L$ was profoundly higher in carcinoma cells compared to surrounding epithelial cells (Figure 1C). Furthermore, Mcl-1 expression was also (slightly) higher compared to surrounding epithelial cells in all probes tested ($n = 4$).

Sensitivity of Bcl-x$_L$ and Mcl-1 expressing CRC cells towards chemotherapeutic drug- induced apoptosis and EGFR1 inhibition

Subsequently, we tested the sensitivity of CRC cell lines towards chemotherapeutic drug-induced apoptosis. We treated SW480 cells with different agents frequently applied for the treatment of patients with CRC: the chemotherapeutic agents irinotecan, oxaliplatin and 5-FU (Figure 2). After 48 h, oxaliplatin (10 μg/mL) and irinotecan (40 μg/mL) induced apoptosis in more than 50% of the cells. 5-FU (10 μg/mL) induced apoptosis in nearly 45% of CRC cells. Treatment with the antagonistic EGFR1 antibody cetuximab induced apoptosis in 18% of cells after 48 h (compared to 13% of apoptosis in control cells, $P = 0.07$, Figure 2). Apoptosis induction in cells treated with the EGFR1 tyrosine kinase inhibitor PD168393 was not significant (16% *vs* 13%, $P = 0.15$).

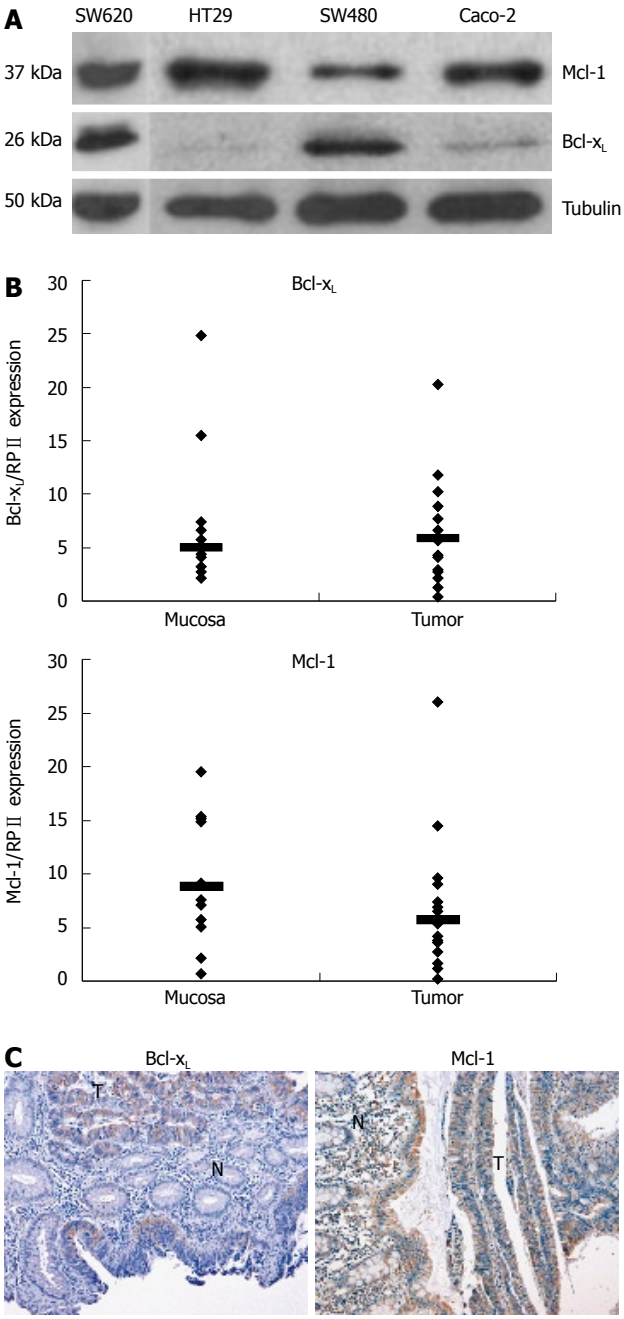

Figure 1 Mcl-1 and Bcl-x$_L$ expression in CRC. **A**: The CRC cell lines, HT29, SW620, SW480, and Caco-2, were analyzed for the basal expression of the Bcl-2 family members Bcl-x$_L$ and Mcl-1. Whole cell lysates were prepared, separated, and immunoblotted with antibodies against Bcl-x$_L$, Mcl-1 and α-tubulin; **B**: CRC tissues and normal colorectal tissues were tested for mRNA expression ($n = 9$ patients). mRNA expression levels of *Bcl-x$_L$*, *Mcl-1* and *RP II* were measured in all tissue samples by quantitative real-time PCR. mRNA expression levels of *Bcl-x$_L$* or *Mcl-1* were normalized to *RP II* in each sample. Each PCR reaction was run in triplicates. Median is added; **C**: Immunohistochemical analysis of human CRC tissues was performed as described in the Methods section. All sections included carcinoma as well as normal epithelial tissues to directly compare Bcl-x$_L$ as well as Mcl-1 expression in neoplastic and non-malignant tissues. Representative analysis of immunoperoxidase detection of Bcl-x$_L$ and Mcl-1 in paraffin embedded carcinoma tissue (T) and adjacent non-tumor tissue (N) is presented.

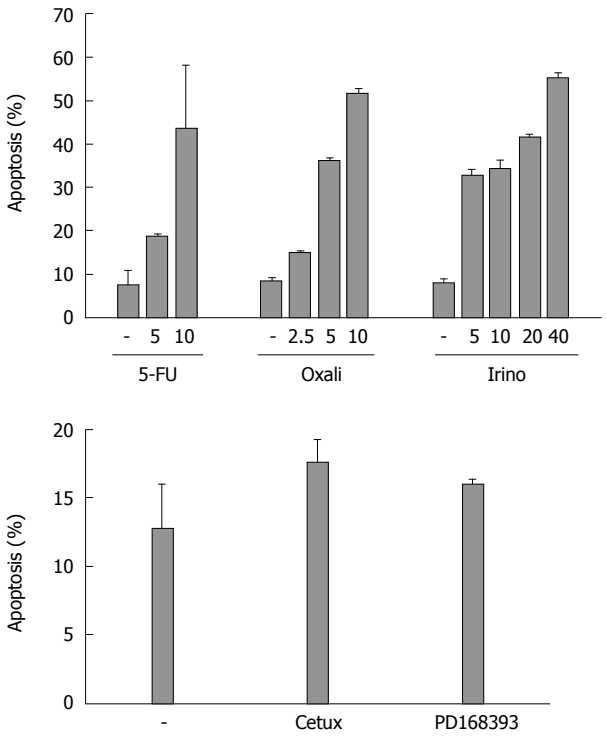

Figure 2 Drug-induced apoptosis in CRC cells. SW480 cells were treated with the chemotherapeutic agents 5-FU, oxalipatin ("oxali") and irinotecan ("irino") for 48 h (concentrations as indicated, upper panel). In addition, cells were treated with the EGFR1 antibody cetuximab ("cetux", 20 μg/mL) or the EGFR1 tyrosine kinase inhibitor PD168393 (7 μmol/L, lower panel) in FCS reduced medium. Cells were then harvested and analyzed for apoptosis induction by flow cytometry. Assays were performed in triplicates. Values are mean ± SD.

Modulation of Bcl-x_L and Mcl-1 expression and its impact on chemotherapeutic drug-induced apoptosis

In order to analyze the functional contribution of Bcl-x_L and Mcl-1 on apoptosis sensitivity of CRC cells, we specifically modulated their expression. Transfection of specific siRNA sequences effectively knocked down expression of Bcl-x_L and Mcl-1 mRNA and protein in SW480 cells (Figures 3A and 4A). For example, 24 h after transfecting 20 nmol/L of specific siRNAs, mRNA expressions were reduced by 75% or 74%, respectively. For 10, 20 and 40 nmol/L of siRNA concentration, an efficient knock down of *Bcl-x_L* and *Mcl-1* was observed after 24 h (data not shown). As could be detected in Western blot assays, Bcl-x_L and Mcl-1 protein expression were also drastically reduced 24 h after siRNA transfection. On the other hand, transfection of expression plasmids for Bcl-x_L increased protein expression (Figure 3C). Next, we tested the effect of specific knock down of Mcl-1 *vs* Bcl-x_L on apoptosis sensitivity of SW480 cells. Knock down of Bcl-x_L significantly enhanced apoptosis induction in untreated cells compared to control transfected cells (21% to 38%, $P < 0.05$, Figure 3B). These results were confirmed in viability assays: Viability was decreased by 33% (Figure 3B). In contrast, Mcl-1 knock down only moderately enhanced spontaneous apoptosis rates (20% to 25%, $P < 0.05$, Figure 4B). Correspondingly, viability was not decreased in CRC cells after Mcl-1 knock down (Figure 4B).

Subsequently, we treated CRC cells with the chemotherapeutic agents 5-FU, oxaliplatin and irinotecan. Knock down of Bcl-x_L in combination with chemotherapy resulted in an increase of apoptosis induction (5-FU: 34% (chemotherapy alone) to 53% (chemotherapy plus Bcl-x_L knock down); oxaliplatin: 35% to 47% and irinotecan: 58% to 72%, $P < 0.05$, Figure 3B). Silencing of Mcl-1 expression led to a slight enhancement of irinotecan-induced apoptosis (33% to 35%, not significant, Figure 4B) and to a moderate, but significant increase of oxaliplatin-induced apoptosis (27% to 33%, $P < 0.05$). Surprisingly, 5-FU-induced apoptosis was decreased in cells with lower Mcl-1 expression (32% to 28%, not significant, Figure 4B). Transfection of the Bcl-x_L expression plasmid enhanced viability of CRC cells: spontaneous apoptosis rates were 20% instead of 36% in control transfected cells ($P < 0.05$, Figure 3C). Moreover, 5-FU (33% *vs* 46%, $P < 0.05$), and irinotecan (26% *vs* 34%, $P < 0.05$)-induced apoptosis, but not oxaliplatin-induced apoptosis (29% *vs* 30%, n.s.), was reduced by transfecting Bcl-x_L (Figure 3C). After transfection of Mcl-1 expression plasmids, no significant impact on chemotherapeutic-drug-induced apoptosis was observed (data not shown).

Modulation of Bcl-x_L and Mcl-1 expression and its impact on EGFR1 blockage

We next analyzed apoptosis induction in CRC cells after targeted therapy approaches. Inhibition of EGFR1 signalling has already entered clinical routine in the treatment of patients with CRC. Cetuximab, a monoclonal antibody against EGFR1, demonstrates anti-tumor efficacy both as a single agent and in combination with irinotecan- and oxaliplatin-based chemotherapy. EGF is known to contribute to an apoptosis resistant phenotype of carcinoma cells. First, we tested the influence of EGF treatment on Bcl-x_L and Mcl-1 expression *in vitro*. EGF treatment of CRC cells induced expression of Bcl-x_L and Mcl-1 after 1.5 and 2.5 h, respectively (Figure 5A). We next analyzed the role of Mcl-1 and Bcl-x_L for the resistance towards targeted therapy. First, we treated CRC cells with cetuximab. Treatment with cetuximab alone (100 μg/mL) did not induce apoptosis SW480 cells after 24 h (Figure 5B). However, in cells with reduced Bcl-x_L, a significant increase in apoptosis induction after treatment with cetuximab (44% *vs* 37%, $P < 0.05$, Figure 5B) or the EGFR1 tyrosine kinase inhibitor PD168393 (0.7 μmol/L; 41% *vs* 29%, $P < 0.01$, Figure 5B) was observed. Moreover, knock down of Mcl-1 also resulted in a moderate, but significant sensitization towards cetuximab (28% *vs* 23%; Figure 5B, $P < 0.01$) and PD168393 (32% *vs* 24%, Figure 5B, $P < 0.05$).

Modulation of Bcl-x_L and Mcl-1 expression and its impact on TRAIL- and CD95-mediated apoptosis

The death receptor ligand TRAIL is a promising anticancer agent (for recent review[21]) and already has been tested in clinical studies in CRC patients. Thus, we analyzed the impact of Bcl-x_L and Mcl-1 modulation

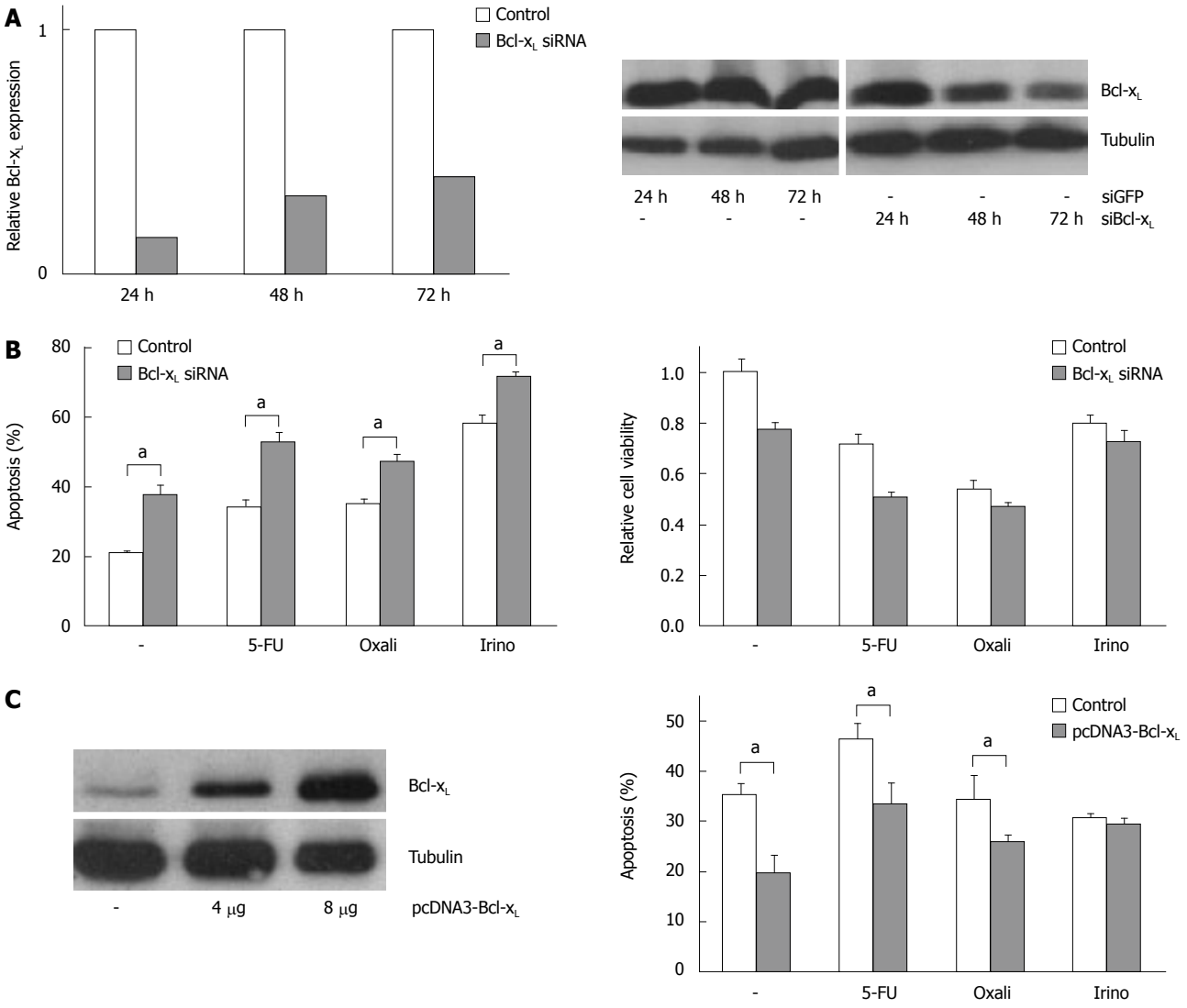

Figure 3 Modulation of Bcl-x_L expression alters chemotherapeutic drug-induced apoptosis. **A**: SW480 cells were transfected with siRNA specific for Bcl-x_L or transfected with siRNA specific for GFP as control (20 nmol/L). After the indicated time post transfection, total RNA was extracted and analyzed for Bcl-x_L expression by quantitative real-time PCR (left panel). Relative expression was calculated as described in the materials and methods section. In addition, after the indicated time post transfection, cells were lyzed and analyzed for Bcl-x_L expression by Western blot (right panel). α-Tubulin expression was used to control equal loading; **B**: SW480 cells were transfected with siRNA specific for Bcl-x_L or transfected with siRNA specific for GFP as control. 24 h post transfection, cells were treated with 5-FU (10 μg/mL), oxaliplatin (16 μg/mL) and irinotecan (60 μg/mL) for further 24 h. Cells were then harvested and analyzed for apoptosis induction (left panel). In addition, cell viability was measured by MTT assay and is shown relative to mock treated controls (right panel); C: SW480 cells were transfected with pcDNA3 Bcl-x_L or pcDNA3 empty vector as control. 24 h post transfection, cells were analyzed for Bcl-x_L expression using Western blotting (left panel). In addition, 24 h post transfection, cells were treated with 5-FU (15 μg/mL), oxaliplatin (10 μg/mL) and irinotecan (40 μg/mL) for further 24 h. Cells were then harvested and analyzed for apoptosis induction by flow cytometry. (B) and (C) assays were performed in triplicates. Values are means ± SD, $^aP < 0.05$.

on TRAIL-induced apoptosis of CRC cells. TRAIL efficiently induced apoptosis in SW480 cells (22% *vs* 3% apoptosis in control cells, $P < 0.05$, Figure 6A). Importantly, Bcl-x_L downregulation enhanced TRAIL-induced apoptosis more than two-fold (46% *vs* 22%, $P < 0.001$, Figure 6A). To further evaluate the sensitizing effect of Bcl-x_L knock down for TRAIL efficacy, we included another CRC cell line, SW620, in our study. After Bcl-x_L knock down, TRAIL-induced apoptosis was increased from 16% to 27% ($P < 0.05$, Figure 6A). Mcl-1 knock down sensitized SW480 cells to TRAIL (17% *vs* 11%, $P < 0.05$, Figure 6B). Upregulation of Bcl-x_L by transfection of expression plasmids decreased TRAIL-induced cell death ($P < 0.05$, Figure 6C). Subsequently, we tested the effect of Bcl-x_L and Mcl-1 knock down on CD95-induced apoptosis of CRC cells (Figure 6D). SW480 cells were highly susceptible to CD95 stimulation by the agonistic CD95 antibody anti-APO-1 (65% *vs* 3% after 24 h of anti-APO-1 treatment, Figure 6D). Bcl-x_L knock down further increased anti-APO-1 induced apoptosis from 65% to 80% ($P < 0.001$). Mcl-1 knock down did not further enhance CD95-triggered apoptosis of CRC cells (Figure 6D).

DISCUSSION

In the present study we demonstrate an important role of the anti-apoptotic Bcl-2 family members Bcl-x_L and, to a lower extent, Mcl-1, for the apoptosis sensitivity of CRC cells. Bcl-x_L expression is considerably enhanced in

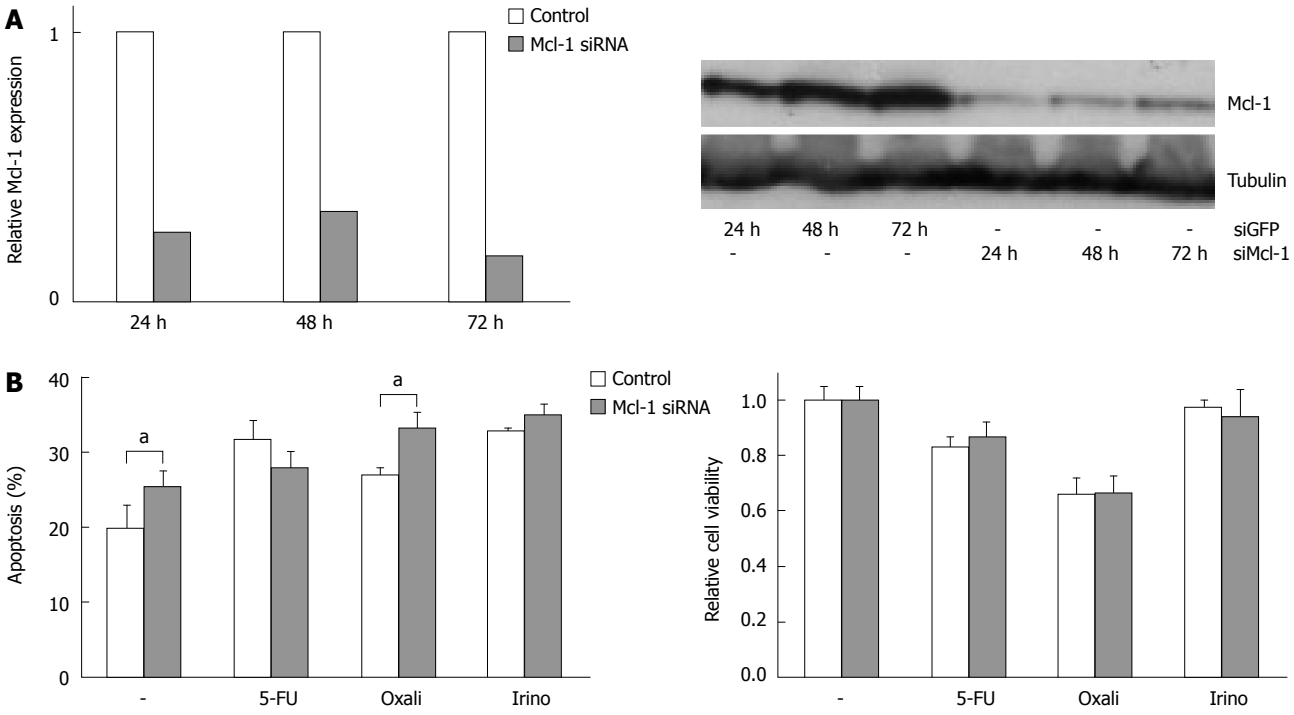

Figure 4 Modulation of Mcl-1 expression alters chemotherapeutic drug-induced apoptosis. **A**: SW480 cells were transfected with siRNA specific for Mcl-1, or transfected with siRNA specific for GFP as control (20 nmol/L). After the indicated time post transfection, total RNA was extracted and analyzed for Mcl-1 expression by quantitative real-time PCR (left panel). Relative expression was calculated as described in the materials and methods section. In addition, after the indicated time post transfection, cells were lyzed and analyzed for Mcl-1 expression by Western blot (right panel). α-Tubulin expression was used to control equal loading; **B**: SW480 cells were transfected with siRNA specific for Mcl-1 or transfected with control siRNA. 24 h post transfection cells were treated with 5-FU (10 μg/mL), oxaliplatin (16 μg/mL) and irinotecan (60 μg/mL) for further 24 h. Apoptosis induction was measured by flow cytometry (left panel) and MTT assay (right panel). Values are mean ± SD, $^aP < 0.05$.

CRC tissue compared to adjacent non-malignant tissue. After knock down of Bcl-x_L by RNA interference, CRC cells prove to be more sensitive towards chemotherapy, EGFR1 blockage, CD95 triggering and treatment with the death receptor ligand TRAIL. The sensitizing effect of Mcl-1 knock down is comparatively moderate. Our data suggest, that Bcl-2 family members, such as Bcl-x_L, are promising targets to improve treatment of patients with CRC. Since strategies to inhibit Bcl-x_L activity have already been applied in preclinical studies, our data are of particular interest[22].

In the past two decades, substantial progress has been made in the treatment of colon cancer, the second most common cancer in western countries. However, therapy resistance of CRC remains a common clinical problem, so that recurrence and metastasis of CRC remain major obstacles in oncology. Thus, new strategies to overcome resistance to current treatment options are needed.

Numerous defects in apoptosis signalling have been described in CRC. These defects appear to be involved in colorectal tumorigenesis, by facilitating tumor cell progression[23]. In addition, defects in apoptosis signalling represent principle mechanisms through which cancer cells are enabled to survive therapy, since chemotherapy and irradiation induce cell death mainly by apoptosis induction[24]. These defects include, among others, stabilization of mitochondria, inactivation of death receptor signalling and overexpression of EGFR1[2].

Anti-apoptotic proteins of the Bcl-2 family, such as Bcl-2, Bcl-x_L and Mcl-1 critically regulate mitochondrial integrity. Increased expression of anti-apoptotic Bcl-2 proteins counteracts chemotherapeutic drug-induced apoptosis in cancer cells. In our study, human CRC tissues revealed enhanced Bcl-x_L expression both on mRNA and protein levels. In line with our study, previous studies also observed enhanced Bcl-x_L expression in CRC tissues[13,25]. In contrary, Northern blot as well as immunohistochemical analysis failed to detect Bcl-2 expression in CRC[25]. The reason for increased Bcl-x_L expression in CRC remains elusive. However, a consistent finding is that oncogenic tyrosine kinases induce expression of Bcl-x_L and enhance protein stability. Among these tyrosine kinases is EGFR1, which has been described to upregulate Bcl-x_L in other tumor models[26]. In line with this observation, we show a significant upregulation of Bcl-x_L in CRC cells upon treatment with EGF in this study. Since overexpression of EGFR1 is a frequent finding in CRC cells, EGFR1 signalling may represent a major cause for the induction of Bcl-x_L expression in CRC. Another mechanism which triggers Bcl-x_L activity in tumor cells is suppression of deamidation[27]. In a recent immunohistochemical evaluation of human CRC tissues, hypoxia-inducible factor-1 has been discussed to induce expression of Bcl-x_L[28].

We also explored expression of the anti-apoptotic protein Mcl-1 in CRC. In other cancer entities, e.g. in hepatocellular carcinoma, a significant correlation of

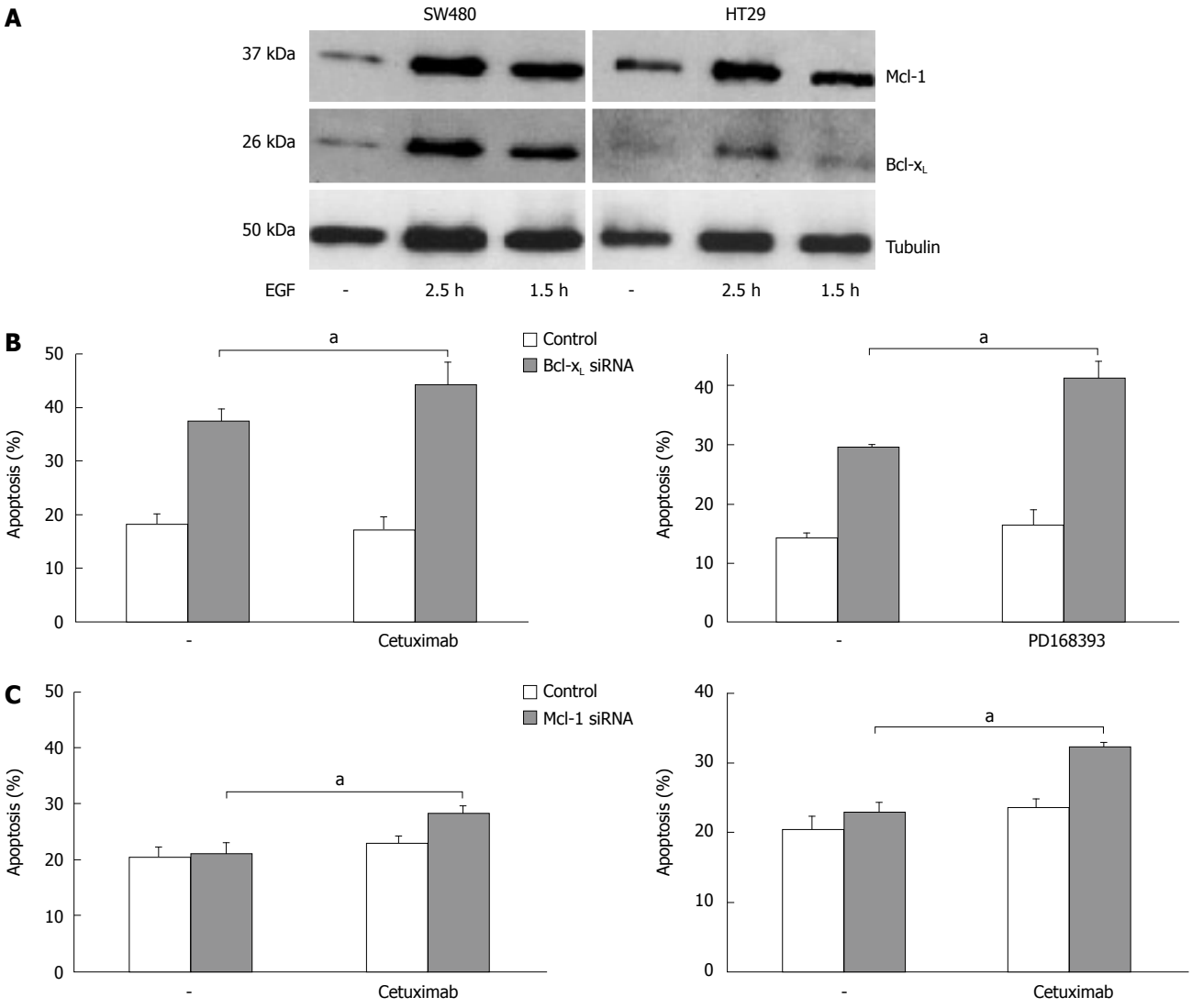

Figure 5 Bcl-x_L and Mcl-1 knock down enhances apoptosis induction after EGFR1 inhibition. **A**: SW480 and HT29 cells were treated with EGF (100 ng/mL) for the time indicated. Whole cell lysates were prepared, separated, and immunoblotted with antibodies against Bcl-x_L, Mcl-1 and α-tubulin; **B**: SW480 cells were transfected with siRNA specific for Bcl-x_L (upper panel), or Mcl-1 (lower panel), respectively, or transfected with siRNA specific for GFP as control. 24 h post transfection cells were treated with cetuximab (100 μg/mL) or PD168393 (0.7 μmol/L) for further 24 h. Cells were then harvested and analyzed for apoptosis induction by flow cytometry. Assays were performed in triplicates and are representative for at least two independent experiments. Values are means ± SD, $^aP < 0.05$.

Bcl-x_L and Mcl-1 with apoptosis resistance was observed[29]. In our study, *Mcl-1* mRNA expression was significantly lower in CRC tissue compared to non-neoplastic cells. In contrast, no profound difference was observed on protein level in immunohistochemistry. Previous studies, however, observed decreased Mcl-1 expression relative to normal mucosa and adenomas, also in immunohistochemistry[13]. At the same time, decrease in Mcl-1 expression has been discussed as a later event in the progression of colorectal tumors, since adenomas show no decreased Mcl-1 expression[13]. The significant difference in *Mcl-1* expression we observed on mRNA level, is in line with these previously published data. Another important aspect about Mcl-1 expression is the staining pattern: in contrast to normal mucosa cells, Mcl-1 has been described to be diffusely expressed in tumour samples in previous studies[30]. Such a diffuse expression could also be detected in our study.

Next, we confirmed that Mcl-1 has a relatively short half-life in CRC cells. Inhibition of translational events in different CRC cell lines resulted in a much more rapid decrease in Mcl-1 compared to Bcl-x_L expression. This is in line with the well-known fact that Mcl-1 provides short-term cell viability protection against cell death during critical transitions in the cell fate[6]. The rapid decrease of Mcl-1 expression can be explained mainly by efficient proteasomal degradation. In addition, we could show that Mcl-1 expression is induced upon treatment with EGF. This might be mediated at least in part by activation of the MEK/ERK-pathway. In lung cancer, it has been shown that EGF enhanced Mcl-1 protein level in an ERK-dependent manner[8]. In our study, EGF also induced expression of Bcl-x_L and ERK might also be involved in this context. Chemotherapeutic agents such as oxaliplatin have been shown to decrease anti-apoptotic proteins like Bcl-x_L in SW480 cells[31]. This effect at least in part explains the apoptosis-inducing capacity of oxaliplatin. However, other chemotherapeutic agents such as

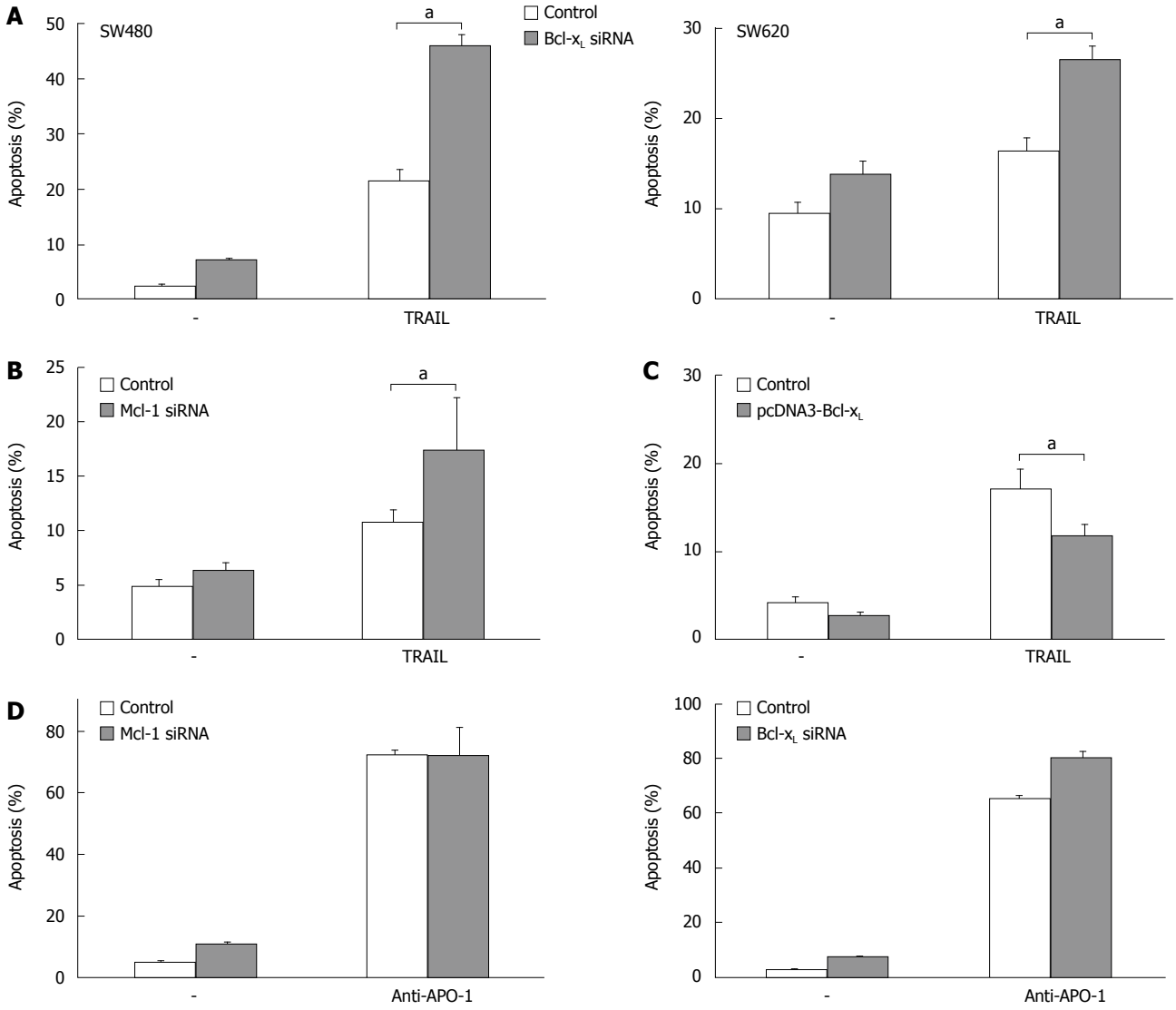

Figure 6 TRAIL- and CD95-induced apoptosis after modulation of Mcl-1 and Bcl-x_L expression. **A**: SW480 or SW620 cells were transfected with siRNA specific for Bcl-x_L or GFP as control. 24 h post transfection cells were treated with TRAIL (0.1 μg/mL) for further 24 h. Cells were then harvested and analyzed for apoptosis induction by flow cytometry; **B**: SW480 cells were transfected with siRNA specific for Mcl-1 or GFP as control. 24 h post transfection cells were treated with TRAIL (0.01 μg/mL) for further 24 h. Cells were then harvested and analyzed for apoptosis induction by flow cytometry; **C**: SW480 cells were transfected with pcDNA3 Bcl-x_L or pcDNA3 empty vector as control. 24 h post transfection, cells were treated with TRAIL (0.1 μg/mL) for further 12 h. Cells were then harvested and analyzed for apoptosis induction by flow cytometry; **D**: SW480 cells were transfected with siRNA specific for Mcl-1 (left panel) or Bcl-x_L (right panel) or with control siRNA. 24 h post transfection cells were treated with anti-APO-1 (0.1 μg/mL) for further 24 h. Cells were then harvested and analyzed for apoptosis induction by flow cytometry. All assays were performed in triplicates and are representative for at least three independent experiments. Values are mean ± SD, $^aP < 0.05$.

5-FU and paclitaxel did not downregulate Bcl-x_L in CRC cells in previous studies[32].

Our results demonstrate that a specific knock down of Bcl-x_L and, to a lower extent, knock down of Mcl-1 by RNAi sensitize CRC cells to chemotherapeutic drugs frequently applied in CRC therapy (5-FU, oxaliplatin and irinotecan). Notably, Bcl-x_L knock down alone already significantly induces apoptosis in untreated cells. Thus, Bcl-x_L expression is important for the survival of CRC cells. Our results extend studies which have already shown that knock down of Bcl-x_L effectively blocks proliferation of CRC cells[33]. On the other hand, overexpression of Bcl-x_L counteracts chemotherapeutic drug-induced apoptosis. In previous studies, overexpression of Bcl-x_L has been reported to enhance resistance to various chemotherapeutic agents in leukaemia cells[34]. However, in a previous study, resistance of CRC cells was not observed in Bcl-x_L overexpressing cells treated with 5-FU or TRAIL[33] In our study, Bcl-x_L overexpression only reduced apoptosis rates in CRC cells treated with 5-FU, irinotecan and oxaliplatin.

Bcl-x_L exerts anti-apoptotic effects in cancer cells mainly by its interaction with pro-apoptotic Bcl-2 family members, e.g. Bax and Bak. Activation of Bax and Bak commit the cell to apoptosis by permeabilizing the outer mitochondrial membrane. However, interaction with Bcl-2 proteins such as Bcl-x_L and Mcl-1 ablates pro-survival functions of Bax and Bak[35]. Bax expression is not reduced in CRC tissues[25]. Thus, Bcl-x_L silencing might induce apoptosis *via* release of Bax and concomitant mitochondrial permeabilization. However, more studies are required to fully understand the roles of the

Bcl-2 proteins and how they cooperate to regulate CRC cell survival.

The apoptosis-sensitizing effect of Mcl-1 modulation was less pronounced (e.g. for oxaliplatin and irinotecan-induced apoptosis) or not significant (e.g. for 5-FU-induced apoptosis), respectively. The reason might be the relatively low expression of Mcl-1 in CRC cells, which may at least in part be compensated by a higher Bcl-x_L expression. Nevertheless, Mcl-1 knock down has sensitizing effects in our study. This effect may also be explained by the fact that Mcl-1, like Bcl-x_L, guards Bax and Bak and thereby prevents them to activate mitochondria.

Notably, Bcl-x_L knock down by siRNA also enhances death receptor-mediated apoptosis in CRC cells. CD95-mediated apoptosis was increased by Bcl-x_L knock down in SW480 cells. However, this effect is supposed to be less relevant for the *in vivo* situation, since no CD95 expression has been detected in CRC tissues in previous studies[30]. Remarkably, Bcl-x_L knock down considerably enhanced tumor necrosis factor alpha (TNF-alpha)-related apoptosis-inducing ligand (TRAIL)-induced apoptosis of SW480 as well as SW620 cells. TRAIL is a member of the TNF family, which has been reported to induce apoptosis in various tumor cells, but not in normal cells, thus representing a promising anticancer agent[36]. Agonistic TRAIL receptor antibodies have already entered clinical trials[37]. Recently, we have shown that treatment with TRAIL alone or in combination with chemotherapeutic drugs (with the exception of cisplatin) is not toxic for human hepatocytes[38]. In our study, treatment of CRC cells with TRAIL resulted in relatively low apoptosis rates. This is in line with previous studies on CRC cells, e.g. on SW620 cells[39] or HT29 cells[40]. The reason for restricted apoptosis induction is that TRAIL signalling also involves activation of anti-apoptotic pathways including PI3K/Akt, NF-κB and MEK/ERK[41]. NF-κB activation, for example, induces anti-apoptotic proteins such as Mcl-1 in HT29 cells, contributing to TRAIL resistance[39]. Bcl-x_L has also been shown to be upregulated by NF-κB activity[42].

Several approaches have been exploited to sensitize cancer cells towards TRAIL. An important strategy, also pursued in this study, is downregulation of anti-apoptotic Bcl-2 proteins. In the present study, Bcl-x_L knock down by siRNA efficiently sensitized CRC cells towards TRAIL-induced apoptosis. In contrary, Mcl-1 knock down only slightly sensitized CRC cells to TRAIL. These data correspond to previous studies of our group and others on hepatocellular carcinoma, where Mcl-1 knock down did not sensitize towards TRAIL[9]. However, in cholangiocellular carcinoma, where high Mcl-1 expression is frequently found, Mcl-1 knock down renders cancer cells susceptible to TRAIL[11]. Since TRAIL-induced apoptosis in cancer cells is hampered by NF-κB activation, inhibition of NF-κB is likely to augment TRAIL-induced death of CRC cells. Approaches to block NF-κB are, among others, peptidomimetic compounds that disrupt the IKK complex or multikinase inhibitors, such as sorafenib[43].

The epidermal growth factor receptor (EGFR1) is a receptor tyrosine kinase of the ErbB family that is abnormally activated in many epithelial tumors, such as CRC. EGFR1 is involved in survival signalling, cell migration, metastasis formation, angiogenesis, and reduced responses to chemotherapy. Clinical and survival benefits with anti-EGFR1 agents have been demonstrated in tumor patients (for review[44]). Monoclonal antibodies to EGFR1 are among promising novel targeted therapies being explored in CRC. One such agent that inhibits EGFR1 signalling by interfering with ligand-binding is cetuximab. Cetuximab is a human-mouse chimeric therapeutic monoclonal antibody that competitively binds to the extracellular domain of EGFR1. EGFR1 tyrosine kinase inhibitors, such as PD168393, also block EGFR1 signalling. In this study, knock down of Mcl-1 slightly sensitized CRC cells towards cetuximab and PD168393. Moreover, knock down of Bcl-x_L sensitized CRC cells towards cetuximab as well as PD168393. These findings suggest that combining EGFR1 blockage with agents that directly destabilize or disable Bcl-x_L and Mcl-1 will have therapeutic benefits.

The development of siRNA technology has made it possible to suppress the function of specific molecules and helps to develop new treatment strategies for cancer[45]. Our study suggests that Bcl-x_L and Mcl-1 are suitable targets to sensitize CRC cells to death. The delivery of siRNA *in vivo* including specific uptake in tumor cells remains a challenging issue[46]. Many approaches use plasmid and viral vectors for transcription of short-hairpin RNAs, both *in vitro* and *in vivo*. However, human trials are still on the way to optimize delivery techniques. Another approach to specifically knock down Bcl-x_L expression is by antisense oligonucleotides. Bispecific antisense oligonucleotides inhibiting both Bcl-2 and Bcl-x_L may be useful to induce apoptosis of tumor cells[47]. Other promising strategies to downregulate Bcl-x_L or Mcl-1 are application of small-molecule inhibitors. ABT-737 is an example of one of the first small-molecule inhibitors of Bcl-2/Bcl-x_L shown to be efficacious *in vivo*, causing complete regression in small-cell lung carcinoma tumour xenografts in mice[22]. TW-37 has recently been described to simultaneously inhibit Bcl-2, Bcl-x_L and Mcl-1 in lymphoma cells by targeting the BH3-binding groove of these Bcl-2 proteins[48]. Apart from direct suppression of Bcl-x_L by siRNA or small-molecule inhibitors, suppression of oncogenic tyrosine kinases, such as Src kinases, which trigger Bcl-x_L expression[26], is another promising approach to induce killing of CRC cells.

In conclusion, our findings clearly implicate the anti-apoptotic activity of Bcl-2 family members, such as Bcl-x_L and, to a lower extent, Mcl-1, as important components of the treatment resistance of CRC cells. Efficacy of chemotherapy, EGFR1 blockage and treatment with TRAIL, might be substantially improved by co-suppression of the anti-apoptotic protein Bcl-x_L.

COMMENTS

Background

Colorectal carcinoma (CRC) is a very common malignancy with an increasing

incidence in recent decades. Defects in apoptosis signalling contribute to the resistance of CRC cells towards different treatment regimens. Thus, one of the main goals for oncologic treatment of patients suffering from CRC is to overcome resistance of tumor cells towards apoptosis.

Research frontiers

Decreased sensitivity of mitochondria towards apoptosis stimuli, such as chemotherapy, is a key mechanism for apoptosis resistance of CRC cells. Mitochondrial activation is determined by the interaction of pro- and anti-apoptotic Bcl-2 family proteins, such as Bcl-x_L and Mcl-1. In CRC, anti-apoptotic Bcl-2 family proteins are highly expressed, thus contributing to apoptosis resistance.

Innovations and breakthroughs

In previous articles, the interaction of anti- and pro-apoptotic members of the Bcl-2 protein family and their role for the apoptosis sensitivity of carcinoma cells has been extensively studied. It has been shown that anti-apoptotic Bcl-2 family members are capable of blocking pro-apoptotic members of the family. Approaches to block the activity of anti-apoptotic Bcl-2 proteins, e.g. by RNA interference, have been evaluated and proven to be likely effective for the treatment of cancer patients.

Applications

In this article, authors show an important role of Bcl-x_L and Mcl-1 for the apoptosis resistance of CRC cells. Thus, downregulation of these anti-apoptotic proteins is a promising approach for the treatment of patients with CRC. Here authors show that the use of RNA interference can effectively downregulate Bcl-x_L and Mcl-1 expression in CRC cells. After downregulation, CRC cells are sensitized to chemotherapy and target therapy approaches. Other ways to downregulate these proteins is application of so called "BH3-only mimetics". These drugs can interact with Bcl-x_L and Mcl-1 and thereby induce the release of pro-apoptotic Bcl-2 proteins. "BH3-only mimetics" have already entered clinical trials in cancer patients.

Terminology

Apoptosis is also depicted as programmed cell death. It is characterized by typical morphological alterations, e.g. the condensation of chromatin in the nucleus. Bcl-2 proteins are a large family of proteins, which can be sub-divided in anti-apoptotic members, multidomain pro-apoptotic members and BH3-only pro-apoptotic members. Bcl-x_L and Mcl-1 are both anti-apoptotic members of the Bcl-2 family. Receptors for epidermal growth factor (EGF) contribute to the growth of cancer cells. Therapeutic approaches in patients with CRC target this receptor (e.g. antibodies binding to the EGF receptor as well as small molecules which inhibit the kinase domain of the receptor) and have been proven to be effective anti-cancer reagents in clinical studies.

Peer review

This study shows that Mcl-1 and Bcl-x_L are important anti-apoptotic factors in CRC. Downregulation of Bcl-x_L is proven to be a promising approach to sensitize CRC towards chemotherapy and targeted therapy. Thus, a translational idea for the treatment of CRC is provided. This is a well written paper and the results are important.

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CLINICAL RESEARCH

Staging of portal hypertension and portosystemic shunts using dynamic nuclear medicine investigations

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Abstract

AIM: To explore portal hypertension and portosystemic shunts and to stage chronic liver disease (CLD) based on the pathophysiology of portal hemodynamics.

METHODS: Per-rectal portal scintigraphy (PRPS) was performed on 312 patients with CLD and liver angioscintigraphy (LAS) on 231 of them. The control group included 25 healthy subjects. We developed a new model of PRPS interpretation by introducing two new parameters, the liver transit time (LTT) and the circulation time between right heart and liver (RHLT). LTT for each lobe was used to evaluate the early portal hypertension. RHLT is useful in cirrhosis to detect liver areas missing portal inflow. We calculated the classical per-rectal portal shunt index (PRSI) at PRPS and the hepatic perfusion index (HPI) at LAS.

RESULTS: The normal LTT value was 24 ± 1 s. Abnormal LTT had PPV = 100% for CLD. Twenty-seven non-cirrhotic patients had LTT increased up to 35 s (median 27 s). RHLT (42 ± 1 s) was not related to liver disease. Cirrhosis could be excluded in all patients with PRSI < 5% (P < 0.01). PRSI > 30% had PPV = 100% for cirrhosis. Based on PRPS and LAS we propose the classification of CLD in 5 hemodynamic stages. Stage 0 is normal (LTT = 24 s, PRSI < 5%). In stage 1, LTT is increased, while PRSI remains normal. In stage 2, LTT is decreased between 16 s and 23 s, whereas PRSI is increased between 5% and 10%. In stage 3, PRSI is increased to 10%-30%, and LTT becomes undetectable by PRPS due to the portosystemic shunts. Stage 4 includes the patients with PRSI > 30%. RHLT and HPI were used to subtype stage 4. In our study stage 0 had NPV = 100% for CLD, stage 1 had PPV = 100% for non-cirrhotic CLD, stages 2 and 3 represented the transition from chronic hepatitis to cirrhosis, stage 4 had PPV = 100% for cirrhosis.

CONCLUSION: LTT allows the detection of early portal hypertension and of opening of transhepatic shunts. PRSI is useful in CLD with extrahepatic portosystemic shunts. Our hemodynamic model stages the evolution of portal hypertension and portosystemic shunts. It may be of use in the selection of patients for interferon therapy.

Key words: Chronic liver disease; Portal hypertension; Portosystemic shunts; Per-rectal portal scintigraphy; Angioscintigraphy

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INTRODUCTION

The most frequent causes of chronic liver disease (CLD) are viral infections, ethanol, autoimmune, enzymatic and metabolic disorders[1,2]. Cirrhosis is the final stage of CLD[3]. Liver biopsy is still an important diagnostic tool in CLD[4].

Portal hypertension is a major complication which appears during CLD evolution. It is defined as an increase of portal blood pressure over 5-10 mmHg. Portosystemic shunts open when the venous portal-liver gradient becomes higher than 10-12 mmHg[5,6].

There are inferior, superior, anterior and posterior portosystemic shunts which could communicate with either inferior or superior vena cava territories. The existence of shunts as well as their blood flow is correlated to the severity and prognosis of CLD[7-9].

Investigation of portal pressure and portosystemic shunts may be performed by invasive and non-invasive methods. Invasive techniques offer the most correct data because of the direct measure of portal pressure. They have however a limited clinical use because of risks and costs[10].

Among the non-invasive methods, ultrasonography and upper digestive endoscopy are those currently used. The main parameters measured by ultrasonography to evaluate the effects of increased blood pressure in the portal territory are the diameters of portal, splenic and superior mesenteric veins, together with spleen size and portal flow velocity. However, dilation of portal territory veins can be seen in only 50% of cases[11,12] and only 35%-80% of cirrhotic patients present esophageal varices at upper digestive endoscopy.

Nuclear medicine offers noninvasive static and dynamic procedures to investigate portal hypertension in CLD and to estimate the existence of portosystemic shunts[13].

A classic method is the liver scintigraphy using labeled colloid (planar and SPECT), which offers data regarding portal hypertension by calculating the capture ratio between liver and spleen, respectively between the right and left liver lobes[14,15]. Increased colloid capture in the bone marrow is characteristic for advanced stages of portal hypertension.

Per-rectal portal scintigraphy (PRPS) investigates the hemodynamic importance of portosystemic shunts. Radio-tracer absorbed from rectum passes through inferior mesenteric vein into portal vein-liver-right heart[16-18]. The per-rectal portal shunt index (PRSI) was introduced by the classic works of Shiomi and co-authors as the main parameter calculated at PRPS by analyzing the dynamic curves raised on liver and heart areas[19,20].

Liver angioscintigraphy (LAS) uses the hepatic perfusion index (HPI) to estimate the ratio between the hepatic artery inflow and total liver perfusion, arterial plus portal. Increased HPI (> 40%) in CLD shows the decrease of portal inflow with reactive increase of the flow through the hepatic artery by activation of the buffer response firstly described by Lautt[21,22]. The decrease of portal inflow in advanced CLD is mainly determined by the quantity of blood deviated through portosystemic shunts[23-25]. In cirrhosis, HPI > 100% highlights the reversion of portal flow.

In this study we improved the interpretation of classical PRPS technique allowing a better characterization and staging of portal hypertension and portosystemic shunts.

MATERIALS AND METHODS

PRPS with ^{99m}Tc-pertechnetate was performed in 312 consecutive patients there were 116 females and 196 males between 18 years and 80 years old. Their CLD diagnosis was based on clinical, laboratory, imaging and morphological data. The final diagnoses of the study population are shown in Table 1. The etiology of CLD in 291 patients is presented in Table 2.

LAS was performed on 231 patients with CLD, randomly selected from the 312 explored by PRPS. There were 141 males and 90 females, between 22 years and 77 years old.

Two hundred and four of the 312 patients who underwent PRPS were also investigated by upper digestive endoscopy: 175 of them had cirrhosis. One hundred and eleven of the cirrhotic patients who presented with esophageal varices at upper digestive endoscopy were followed up for 6 mo after investigation.

The control group for PRPS included 25 healthy subjects, 11 females and 14 males, between 18 years and 80 years old. The control group for LAS was composed of 25 healthy subjects, 10 females and 15 males, between 20 years and 78 years old.

PRPS was also performed on one group of 12 patients with complete thrombosis of portal vein in order to calculate RHLT and to compare its values with those in healthy subjects. This group included 6 females and 6 males, between 34 years and 73 years old.

All the patients were fasted for at least 12 h before LAS and PRSI.

Nuclear medicine investigations were made by using a SPECT Orbiter Siemens gamma-camera with high-resolution, low-energy, parallel collimator connected to a Power Macintosh computer, using ICON dedicated software.

For PRPS we used ^{99m}Tc-pertechnetate eluted from Drygen generators (General Electric-Amersham, UK). The colloid used for LAS was Hepatate (General Electric-Amersham, UK) labeled with ^{99m}Tc.

Two enemas were performed in each patient for PRPS: the first on the evening before the exam, and the second two hours prior to the examination.

The patients were positioned at PRPS with the camera detector in the anterior view including the liver

Table 1 Features of the study population according to the final diagnosis (*n*)

Type of disease	Number of patients
Steatosis	17
Chronic hepatitis	69
Cirrhosis	202
Unknown	3
CLD infirmed	21
Total	312

Table 2 Etiology for the patients with chronic liver disease (*n*)

Etiology	Number of patients
Viral	139
Alcohol	74
Mix (viral + alcohol)	6
Unknown	72
Total	291

and heart areas. A solution containing 2 milliliters of ^{99m}Tc-pertechnetate (296-370 MBq) was introduced into the upper part of the rectum, followed by 15 milliliters of air under pressure. Serial scintigrams were recorded every 2 s for 3 min. Radioactivity curves were built on liver and heart areas to show the dynamics of radiotracer absorbed from the rectum.

LAS was performed after antecubital i.v. bolus injection of 370-440 MBq of ^{99m}Tc radio-colloid, with the patients lying down, in anterior-posterior view. Collimator area included abdomen and lower part of thorax. Sequential images were recorded 1/s for 1 min. Right kidney, right liver lobe and spleen dynamic curves were built. HPI was calculated using the Sarper's method[26].

The results were compared with clinical diagnosis, liver biopsy and upper digestive endoscopy. For statistical evaluation of PRSI we used the Kruskall-Wallis and Mann-Withney non-parametric tests.

New approach to the per-rectal portal scintigraphy

At the beginning of the research we used for the first 100 patients the classic interpretation of PRPS, based on the calculation of PRSI for the global area of the liver[16]. All information was stored in the computer.

In the second stage we considered the possibility of acquiring more useful data in PRPS, by developing a new model of interpretation. Pertechnetate is not significantly captured by liver or heart, so that at PRPS the first-passage histograms built for these organs represent transit curves, not accumulation curves. This suggests the importance of time-related parameters. Transit type of PRPS liver and heart curves is highlighted by dynamic curves built on the inferior mesenteric vein area. These curves have the same ascending aspect as the histograms on liver and heart thereafter produced at first passage by the same radiotracer flow absorbed from the upper rectum.

We introduced LTT as a new parameter useful for the early phases of CLD where PRSI offers not enough information. LTT is the time interval between entrance into the liver and subsequent entrance into the right heart of the radiotracer absorbed from the upper rectum, after passing through the mesenteric and portal veins. LTT was separately measured for each liver lobe as the time interval between the liver and heart dynamic histograms. The normal value is 24 ± 1 s. This parameter is useful for patients without extra-hepatic shunts. In patients with portosystemic extrahepatic shunts, LTT cannot be correctly determined by PRPS because the tracer absorbed from the rectum may arrive at the right heart faster by passing through shunts than following the physiological pathway.

PRSI equal to 10% corresponds to LTT equal to 16 ± 1 s. LTT decreased between 16 s and 23 s corresponds to a PRSI increased up to 10% and approximates the interval in which only the transhepatic shunts are open. A PRSI equal to 30% corresponds to a mean time of 8 s between the liver and heart curves, but this interval does not have the significance of LTT because of the flow passing through the extra-hepatic portosystemic shunts, which arrives at the heart faster than the tracer passing through the liver.

Liver areas perfused only through the hepatic artery may be seen in cirrhosis. One lobe or both have, in such cases, abolished or insignificant portal inflow and the time between PRPS curves on the heart and on the area(s) without portal inflow has a maximum value, equal to RHLT. This is a constant time interval (not related to CLD) between the entrance of the tracer into the right heart and its subsequent arrival to the liver following the route: right heart-lungs-left heart-aorta-hepatic artery. We measured RHLT in the patients with complete portal thrombosis as the time interval between heart and liver curves. In healthy controls, RHLT was measured as the time interval between the arrival of tracer into the right heart and the ascending inflexion on the liver histogram determined by the subsequent arrival of tracer through the hepatic artery. The value of RHLT was 42 ± 1 s for both methods.

We performed separate PRPS analyses of the two liver lobes, which are autonomous in relation to the blood inflow (Figure 1). No differences between the two lobes could be found in the control group. We compared PRPS curves for the two lobes in order to distinguish subtypes, respectively stages of portal hypertension evolution. Figure 1 shows the case of a cirrhotic patient in which time intervals between the heart histogram and the curves on the two liver lobes are different.

Our PRPS model is based on the two new time parameters, the classic calculation of PRSI and the separate evaluation of the two liver lobes.

RESULTS

The distribution for the 312 patients of the PRSI calculated using Shiomi's formula is presented in Table 3.

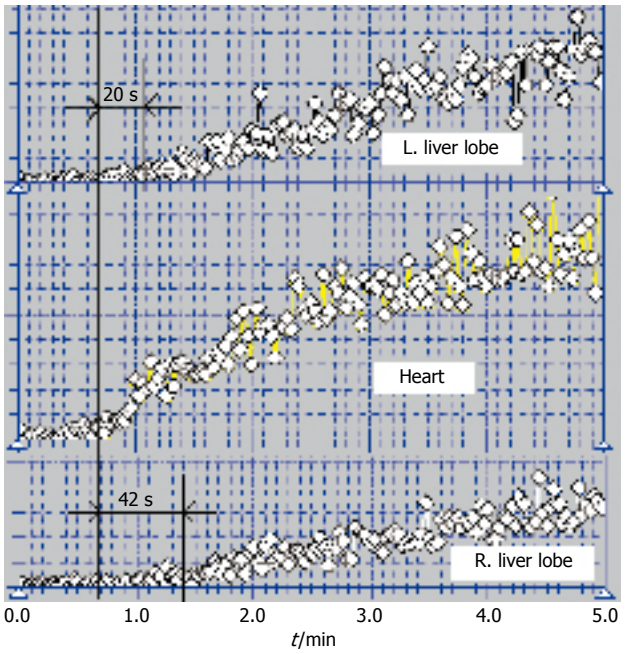

Figure 1 Per-rectal portal scintigraphy dynamic curves. Separate analyses of the two liver lobes for a cirrhotic patient (stage 4b).

There were no correlations of PRPS and LAS parameters with sex or age ($P < 0.01$).

As many as 202 of 312 patients investigated with the PRPS were diagnosed as having a cirrhosis. One hundred and seventy-five of these underwent upper digestive endoscopy. Twenty seven patients with advanced cirrhosis (all of them with PRSI > 30% at PRPS) could not be explored by endoscopy as deemed too risky. Using Child-Pugh classification, the 175 cirrhotic patients investigated by upper digestive endoscopy were classified as follows: 99 in class A, 38 in class B and 38 in class C.

Only 16 patients from the 93 with PRSI between 5% and 30% had esophageal varices. Thirty two additional patients with PRSI < 30% but without varices were also diagnosed with cirrhosis. As a result, we had a total number of 48 cirrhotic patients with PRSI < 30%. Five patients with cirrhosis had discordant low PRSI values ranging from 5% to 10%, but no cirrhotic patient had normal PRSI (< 5%). All the patients with PRSI > 30% had cirrhosis, so we used PRSI = 30% as an upper limit value for chronic hepatitis.

The PRSI was significantly higher in cirrhotic patients than in chronic hepatitis ($P < 0.01$). The median value for PRSI was 5% for the control group, 5% for the patients with steatosis, 6% for the patients with chronic hepatitis and 73.5% for the cirrhotic patients. The sums of ranks for the PRSI values based on Kruskall-Wallis test for the healthy subjects and stages of CLD are shown in Table 4 ($P = 0.000$). Using the Mann-Withney test we showed that there was a significant statistical difference between PRSI for patients with chronic hepatitis and the controls ($P = 0.0003$), between patients with cirrhosis and those with chronic hepatitis ($P = 0.0000$) and respectively between patients with cirrhosis and controls ($P = 0.0000$).

Table 3 Distribution of all the patients and of those with cirrhosis according to the per-rectal portal shunt index and to our classification in 5 stages

Per-rectal portal shunt index (%)	Number of patients (*n*)		Cirrhosis	Stage in our classification
0-5	65		0	Stages 0 & 1
5-10	13		5	Stage 2
10-20	42	80	43	Stage 3
20-30	38			
30-40	17	154	154	Stage 4
40-50	10			
50-60	11			
60-70	10			
70-80	18			
80-90	40			
90-100	48			
Total	312		202	

Table 4 Statistical analysis of the per-rectal portal shunt index for healthy subjects and for the different stages of chronic liver disease using Kruskall-Wallis test

Disease	Number of patients (*n*)	Sum of ranks
CLD infirmed	21	759.5
Steatosis	17	1201.5
Chronic hepatitis	69	5004.5
Cirrhosis	202	40929.5

Kruskall-Wallis test: H (3, $n = 309$) = 169.5024, $P = 0.000$.

One hundred and eleven patients with esophageal varices and PRSI > 30% were followed up for 6 mo; 51 of these had experienced previous upper digestive bleeding. During the follow up, 17 of these patients had an episode of upper digestive bleeding (11 as first time, 6 as recurrence). All our patients with upper digestive bleeding had PRSI > 70% (mean of 88%). The 94 patients from the group of 111 with esophageal varices who had not upper digestive bleeding during the follow up had a mean value of PRSI equal to 46.75%. We had no patients with upper digestive bleeding among those with PRSI < 30%, even if they had esophageal varices at upper digestive endoscopy.

These results show the diagnostic value of the classic parameter PRSI. A PRSI < 5% had a NPV = 100% for cirrhosis, while a PRSI > 30% had PPV = 100% for cirrhosis. A PRSI > 70% was associated with a high risk of upper digestive bleeding ($P < 0.01$), while with a PRSI < 30% no variceal bleeding was encountered. However, using only PRSI is not always possible to make the differential diagnosis between chronic hepatitis and cirrhosis or between chronic hepatitis and healthy subjects.

Using LTT parameter originally introduced by us at PRPS we were able to find more data about the early stages of portal hypertension, when PRSI cannot offer enough information. In healthy subjects, LTT was 24 ± 1 s. The distribution of LTT in patients with PRSI smaller than 10% (LTT > 16 s) is shown in Table 5. For the 27 non-cirrhotic CLD patients who had prolonged LTT

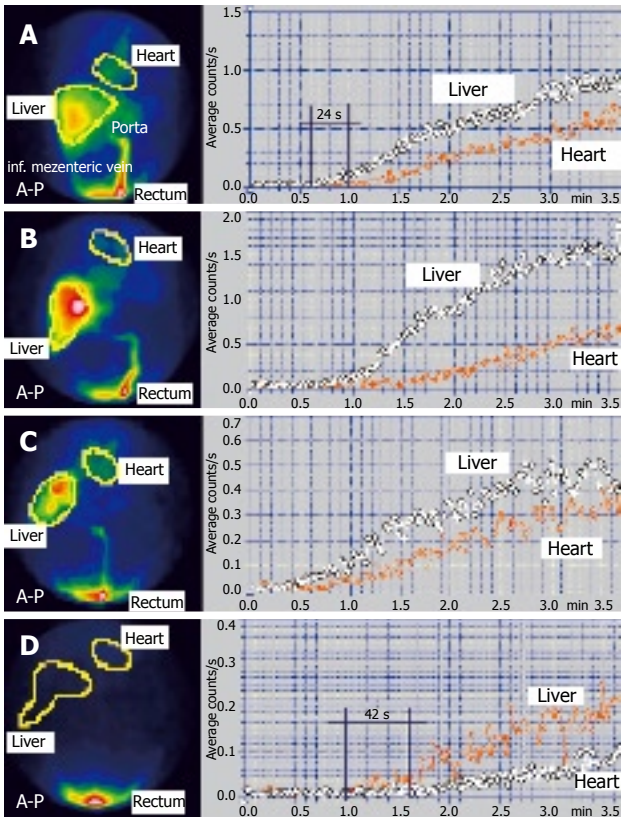

Figure 2 Per-rectal portal scintigraphy. **A**: Stage 0, normal aspect. LTT = 24 s, PRSI < 5%; **B**: Stage 1. LTT increased (33 s). PRSI < 5%; **C**: Stage 2. Decreased LTT (18 s), PRSI slightly increased (8%); **D**: Stage 4c. Time between heart and liver curves is equal to RHTL = 42 s. Cirrhosis with undetectable portal inflow to the both lobes.

Table 5 Liver transit time and distribution in stages of the patients with the per-rectal portal shunt index lower than 10%

Per-rectal shunt index	Mean value of liver transit time (*s*)		Etiology	Number of patients (*n*)	Stage in our classification
	Right lobe	Left lobe			
< 5%	24		-	38	Stage 0
	25	28	viral	10	Stage 1a
	31	24	alcoholic	6	Stage 1b
	31.5	29	viral	7	Stage 1c
			viral + alcoholic	4	
5%-10%	19.5			13	Stage 2

(> 25 s), the median value was 27 s. LTT determined at PRPS has hemodynamic significance as it shows the time required at first-passage by the main part of portal inflow of radio-tracer to arrive to right heart through the liver.

RHLT measured in the control group and in patients with complete portal thrombosis is 42 ± 1 s. We used it to identify cirrhotic patients with undetectable portal inflow to one liver lobe or both. Other causes of portal flow interruption (thromboses, compressions) were excluded by ultrasonography.

HPI was used in our study to show the reactive increase of arterial flow due to the decreasing of portal

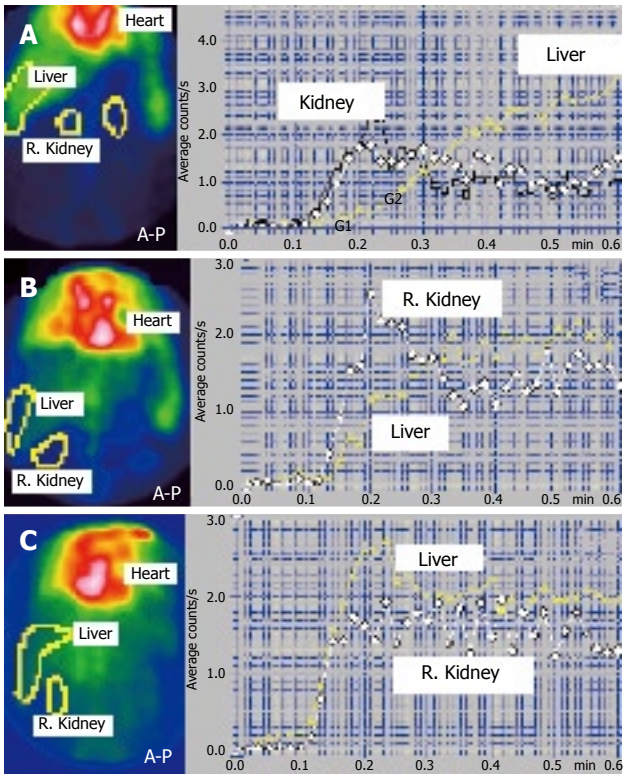

Figure 3 Liver angioscintigrahy. **A**: Normal aspect. HPI = 30%. G1 = arterial inflow segment; G2 = portal inflow segment; **B**: Cirrhotic patient. HPI = 60%; **C**: Cirrhosis with reversed portal flow. HPI = 130%.

inflow (HPI > 40%) and to identify those cirrhotic patients with reversed portal flow (HPI > 100%).

Staging of portal hypertension and portosystemic shunts

Using the above shown data we propose a hemodynamic model in 5 steps to stage the evolution of portal hypertension and portosystemic shunts on physiopathological basis using nuclear medicine dynamic investigations (PRPS and LAS).

The 5 stages of portal hypertension and portosystemic shunts proposed by us are the following: (1) Stage 0 is normal: PRSI < 5%, LTT = 24 ± 1 s (Figure 2A), HPI < 40% (Figure 3A); (2) Stage 1 is characterized by PRSI < 5% (normal), but LTT is increased over 25 s at least on one lobe (Figure 2B). HPI is normal. For stage 1 we found 3 subtypes according to the liver lobe(s) with prolonged LTT: (a) Subtype 1a, with increased LTT for the left lobe, but with normal or slightly decreased LTT for the right lobe; (b) Subtype 1b, with increased LTT for the right lobe and with normal or slightly decreased LTT for the left lobe; (c) Subtype 1c, with increased LTT for the both lobes; (3) Stage 2 is characterized by decreased LTT for both lobes, between 16 s and 23 s. PRSI is slightly increased, between 5% and 10% (Figure 2C). HPI calculated at LAS is currently at the upper normal limit or slightly increased, up to 45%; (4) Stage 3 is characterized by a moderately increased PRSI, between 10%-30%. Time interval between the hepatic and heart curves is decreased between 8 s and 16 s, but it is no more equal to LTT due

to the shunts. HPI is moderately increased, usually up to 50%-55%; (5) Stage 4 is characterized by PRSI > 30%. HPI is increased over 60%-70%. Liver curve precedes heart curve with less than 8 s or heart curve precedes liver curve (when PRSI > 50%). We found 4 subtypes of stage 4, according to the lobe(s) with undetectable or reversed portal flow: (a) Subtype 4a: heart curve precedes the hepatic histograms on both lobes with less than 42 s. Both lobes have portal inflow; (b) Subtype 4b: Cardiac curve precedes right liver lobe histogram with 42 s and the left liver lobe histogram with less than 42 s. There is still a portal flow to the left lobe, but the portal inflow to the right lobe is undetectable (Figure 1); (c) Subtype 4c: PRSI > 95%. Time between heart and both liver lobes curves is equal to RHLT = 42 s (Figure 2D). PRPS cannot detect portal inflow to any of the hepatic lobes. For subtypes 4a, 4b and 4c, the HPI is increased over 60%, but smaller than 100% (Figure 3B); (d) Subtype 4d: HPI at LAS is higher than 100% (Figure 3C). The time between PRPS heart curve and both liver lobes histograms is 42 s. Portal flow is reversed.

DISCUSSION

Stage 0 includes the subjects without CLD. There are no portal flow changes. In our study, stage 0 had NPV = 100% for CLD. The tracer absorbed at PRPS from the rectum reaches the liver through the physiological pathway (inferior mesenteric and portal veins).

In stage 1, it is possible to detect the earliest changes that affect either one lobe or both, determined by the increased resistance opposed by liver to portal inflow. Portal flow velocity decreases and LTT is consequently increased. In our study stage 1 had PPV = 100% for non-cirrhotic CLD. In this stage the transhepatic and extrahepatic shunts are not open and the arterial inflow is normal. We encountered subtype 1a in patients with chronic viral hepatitis, subtype 1b in alcoholic etiology and subtype 1c in viral and mix (viral + alcohol) etiologies.

Stage 2 theoretically corresponds to the dilation of part of transhepatic pathways between portal and hepatic veins as a result of the portal pressure which is increased at higher values than in stage 1. The blood passes faster through these dilated transhepatic shunts than through sinusoids[27,28]. LTT is consequently decreased. Our threshold PRSI = 10% between stage 2 and stage 3 was selected based on the correspondence of this PRSI value with LTT = 16 ± 1 s. Extrahepatic shunts are not open and arterial inflow remains normal (hepatic artery buffer response is not activated). Stage 2 theoretically appears when the increased resistance opposed by liver to the portal inflow produces a higher portal pressure which is able to enlarge transhepatic pathways, but is not high enough to open extrahepatic shunts. As stage 1, stage 2 has PPV = 100% for CLD, but stage 2 includes not only chronic hepatitis, but also cirrhotic patients, showing a more advanced stage of portal hypertension than stage 1. 38.46% of our 13 patients in stage 2 had cirrhosis, the other 61.54% had chronic hepatitis.

A steady state between stage 1 and stage 2 may appear in cases with the resistance (and LTT) increased on one more affected liver lobe and with redirecting of an increased percentage of the portal inflow through the other lobe. In such cases, the lobe less affected may encounter opening of transhepatic shunts due to its higher portal inflow (with consequently slightly decreased LTT, like in stage 2), while the more affected lobe has prolonged LTT (characteristic for stage 1).

Stage 3 is theoretically characterized by the opening of extrahepatic shunts, added to the transhepatic dilated pathways already present from stage 2. Low flow extrahepatic shunts appear when the transhepatic shunts are no more able to compensate the higher values of portal pressure. The inferior per-rectal portosystemic shunts are in most cases the first extrahepatic shunts which open. The cause is the pressure gradient between inferior mesenteric vein and inferior vena cava territories, which is lower than the pressure gradient between portal vein and superior vena cava. The portal inflow to the liver decreases due to shunted flow and PRSI increases over 10%. A reactive increase of the arterial liver inflow due to the activation of the buffer mechanism of the hepatic artery is reflected at LAS by HPI > 40%. 53.75% of our 80 patients in stage 3 had cirrhosis, the other 46.25% had chronic hepatitis.

Stage 4 in our study had PPV = 100% for cirrhosis. Theoretically, stage 4 involves shunts open to the territory of superior vena cava, which has a higher diameter and increased flow[29]. PRPS shows diminished or abolished portal inflow to one or both liver lobes, with PRSI > 30%. In stage 4, the tracer absorbed at PRPS from the rectum usually arrives faster to the right heart (through the portosystemic shunts and caval veins) than to the liver through the physiological pathway. Inverted order of heart and liver curves may be thus seen in advanced cases, corresponding to PRSI > 50%. In patients with undetectable portal inflow to one or both lobes (subtypes 4b, 4c, 4d), the tracer absorbed from the rectum reaches those liver areas only through the hepatic artery and time interval between the heart and liver curves is equal to RHLT = 42 s. Subtype 4b appeared more frequently in alcoholic CLD. In our study, we did not have patients with abolished inflow to the left lobe and maintaining portal inflow to the right lobe, but such cases may theoretically exist.

Figure 1 shows a case where the interval between heart and left liver lobe curves was equal to 20 s (left lobe maintained a low portal inflow) while the time interval between heart and right liver lobe histograms was equal to RHLT = 42 s (the right lobe received tracer only through hepatic artery).

In our group, the number of patients in early stages (1 and 2) was lower than in advanced stages (3 and 4). This could be explained by the fact that few patients in early CLD stages were hospitalized and/or proposed for nuclear medicine dynamic liver investigations. Moreover, stage 2 is theoretically an intermediate stage in the natural history of CLD and of the portal hypertension, so it lasts a short time compared to the evolution of the disease.

Our experience confirmed that at PRSI > 70% the risk of upper digestive hemorrhage is increased[17,30].

The classical parameter PRSI allows only a rough characterization of non-cirrhotic patients. PRSI gives no information about early increased resistance opposed by liver to the portal inflow or about the existence of transhepatic shunts, the first that open due to the portal hypertension. Another diagnostic limitation in using PRSI alone is the fact that there are patients with chronic hepatitis but who have normal PRSI, lower than 5%.

Our model using LTT besides PRSI allows a very early diagnosis of portal hypertension, represented by stages 1 and 2. Changes of liver dynamic resistance opposed to portal inflow could be shown in these early stages, at a moment when morphological effects on the portohepatic circulation are not detectable. The diagnosis of early hemodynamic changes determined by portal hypertension could be the basis for an appropriate therapy in a stage when the disease is reversible. Thus, we propose LTT as the main parameter of PRPS in the evaluation of early stages of portal hypertension. PRSI remains a very useful parameter for portal hypertension and portosystemic shunts in advanced stages of CLD.

Staging of portal hypertension has implications in the selection of patients for the treatment. Hemodynamic pathophysiology information offered by nuclear medicine dynamic investigations may improve also the selection of patients for interferon therapy.

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COMMENTS

Background

Portal hypertension and portosystemic shunts are severe complications of chronic liver disease (CLD). Their evaluation could be considered a dynamic marker of the progression of the disease. Ultrasonography and upper digestive endoscopy are usually performed to evaluate their existence and hemodynamic importance. Nuclear medicine techniques like per-rectal portal scintigraphy (PRPS) and liver angioscintigraphy (LAS) can offer valuable supplementary information.

Research frontiers

Doppler ultrasonography and MRI are continuously increasing their accuracy in exploring portal hypertension and portosystemic shunts. PRPS is usually performed to investigate the advanced stages of CLD. The classical PRPS parameter per-rectal portal shunt index (PRSI) is useful in cases with open portosystemic shunts. Our research improves the diagnosis possibilities of PRPS especially in early stages of CLD by introducing two new time parameters. The early diagnosis of CLD and the therapy (including the selection of patients for interferon therapy) may be improved using dynamic scintigraphy data.

Innovations and breakthroughs

We introduced two new parameters at PRPS, respectively liver transit time (LTT) and right heart to liver transit time (RHLT). LTT is useful in early stages of portal hypertension, before the opening of extrahepatic portosystemic shunts. LTT allows the diagnosis of early increase of liver resistance opposed to portal inflow and of the opening of transhepatic shunts. RHLT is useful in advanced CLD stages to detect liver areas missing portal inflow. We propose the classification of portal hypertension and portosystemic shunts in 5 hemodynamic stages, characteristic for the progression of the disease. We introduce the separate evaluation of the two liver lobes at PRPS, used to subtype the stages 1 and 4.

Applications

Using LTT as a basic parameter, PRSI allows the detection of early stages of portal hypertension, which are reversible under proper therapy. Our method can also distinguish between the 1st stage of portal hypertension, with increased resistance opposed by liver to portal inflow, without shunts, and the 2nd stage, characterized by the opening of transhepatic shunts. The new parameter RHLT and the hepatic perfusion index (HPI) calculated at LAS allow a better characterization of liver hemodynamics in advanced cirrhosis. We confirm the results of other studies showing that at PRSI > 70% the risk for upper digestive bleeding increases. The classification of portal hypertension and portosystemic shunts in 5 hemodynamic stages is useful for clinicians, in order to have a more accurate view of the patients with CLD. A better understanding of hemodynamic status of border-line cases between chronic hepatitis and cirrhosis may also improve the selection of patients for interferon therapy. Patients with PRSI between 5%-30% (stages 2 and 3 in our classification) require a precise evaluation in order to choose an adequate therapy. Correlation of dynamic nuclear medicine techniques with other non-invasive methods makes it possible to avoid liver biopsy for guiding the treatment in border-line patients between chronic hepatitis and cirrhosis. Dynamic follow-up of patients under interferon-treatment may be useful to adjust the therapy. Based on calculation of LTT, PRPS may be used to determine whether the early portal pressure reducing effect of anti-viral therapy is maintained in the long term, especially in sustained viral responders. It can be also helpful to evaluate whether long-term use of anti-viral therapy may delay the appearance and decrease the severity of portal hypertension manifestations.

Terminology

Per-rectal portal scintigraphy (PRPS) is a dynamic nuclear medicine technique which investigates the existence and hemodynamic importance of portal hypertension and portosystemic shunts. A radiotracer introduced in the upper rectum is absorbed and follows the next pathway: inferior mesenteric vein-portal vein-liver-right heart. Dynamic curves built on liver and heart allow the calculation of specific parameters. *Liver transit time (LTT)* determined at PRPS is the time interval between entrance into the liver and subsequent entrance into the right heart of the radiotracer absorbed from the rectum, after passing through mesenteric and portal veins. *Right heart to liver transit time (RHLT)* represents at PRPS a constant time interval (not related to liver disease) between the entrance of the tracer into the right heart and its subsequent arrival to the liver following the next route: right heart-lungs-left heart-aorta-hepatic artery. *Per-rectal portal shunt index (PRSI)* is a parameter calculated at PRPS by analysis of dynamic curves built on liver and heart areas. *Liver angioscintigraphy (LAS)* is a dynamic nuclear medicine method based on i.v. antecubital administration of a radio-tracer and subsequent analyses of the liver dynamic curve which is determined at first passage by both hepatic artery and portal inflows of tracer. *Hepatic perfusion index (HPI)* is a parameter calculated at LAS which estimates the ratio between hepatic artery inflow and total liver perfusion, arterial plus portal.

Peer review

This is a well done study probably not completely well presented where Dragoteanu and coworkers used per-rectal portal scintigraphy and liver angioscintigraphy in 312 and 231 CLD patients and 25 controls to calculate hepatic perfusion index (HPI) and other new hemodynamic parameters and classify portal hypertension and porto-caval shunts in 5 hemodynamic stages, which are specifically for the progression of CLD.

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BASIC RESEARCH

Combination of small interfering RNAs mediates greater suppression on hepatitis B virus cccDNA in HepG2.2.15 cells

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Abstract

AIM: To observe the inhibition of hepatitis B virus (HBV) replication and expression in HepG2.2.15 cells by combination of small interfering RNAs (siRNAs).

METHODS: Recombinant plasmid psil-HBV was constructed and transfected into HepG2.2.15 cells. At 48 h, 72 h and 96 h after transfection, culture media were collected and cells were harvested for HBV replication assay. HBsAg and HBeAg in the cell culture medium were detected by enzyme-linked immunoadsorbent assay (ELISA). Intracellular viral DNA and covalently closed circular DNA (cccDNA) were quantified by real-time polymerase chain reaction (PCR). HBV viral mRNA was reverse transcribed and quantified by reverse-transcript PCR (RT-PCR).

RESULTS: siRNAs showed marked anti-HBV effects. siRNAs could specifically inhibit the expression of HBsAg and the replication of HBV DNA in a dose-dependent manner. Furthermore, combination of siRNAs, compared with individual use of each siRNA, exerted a stronger inhibition on antigen expression and viral replication. More importantly, combination of siRNAs significantly suppressed HBV cccDNA amplification.

CONCLUSION: Combination of siRNAs mediates a stronger inhibition on viral replication and antigen expression in HepG2.2.15 cells, especially on cccDNA amplification.

Key words: Combination of small interfering RNAs; Covalently closed circular DNA; Hepatitis B virus; RNA interference; HepG2.2.15 cells

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INTRODUCTION

Hepatitis B virus (HBV) is a major cause for acute and chronic hepatitis in humans. Although recombinant vaccines are widely available, about 400 million people have chronic HBV infection worldwide. Chronic infection may also have serious consequences, and nearly 25% people with chronic HBV infection would die due to untreatable liver cancer[1]. The deaths of liver cancer patients resulting from chronic HBV infection exceeds one million per year worldwide[2]. Therefore, it is important to develop effective strategies for the treatment of HBV-infected patients.

Nucleotide analogues, such as lamivudine, can effectively inhibit HBV DNA synthesis[3,4] and are widely used in the treatment of HBV-infected patients. However, analysis of viral kinetics during lamivudine therapy revealed that a prolonged treatment is required since

lamivudine does not completely inhibit viral replication and the rate of clearance for infected hepatocytes is slow[5]. HBV is not a cytopathogenic virus and hepatocytes are normally long-lived and their half-life is estimated to be 6-12 mo or longer, explaining the requirement for a long-term antiviral treatment course, which is associated with the selection of drug-resistant mutants[6].

During HBV replication, viral covalently closed circular DNA (cccDNA) serves as the template for viral transcription and its production is regulated and amplified by an intra-cellular pathway in which newly synthesized genomic DNA is recycled to the nuclei[7,8]. This process establishes a steady pool of nuclear cccDNA, which is maintained during the life of infected hepatocytes. It is likely that cccDNA may reactivate synthesis of viral transcript and protein, leading to a rebound of active viral replication. Thus, elimination of cccDNA from infected hepatocytes still remains a challenge in therapy for HBV-infected patients. A potent agent used in anti-HBV therapy should be evaluated with special emphasis on its inhibitory effect against the amplification of cccDNA.

Small interfering RNAs (siRNA) are double-stranded RNA molecules, approximately 21 nucleotides in length that hybridize to a homologous mRNA target and result in degradation of mRNA[9]. This posttranscriptional gene silencing process, called RNA interference (RNAi), is evolutionarily conserved in both plants and eukaryotic cells[10,11]. Due to the high sequence specificity and efficiency, RNAi can be used in functional genomic studies such as human immunodeficiency virus (HIV), hepatitis C virus (HCV), and influenza virus, *etc*[12-14]. HBV is one of the major candidates for RNAi, as its pregenomic RNA is a key intermediate for maintaining DNA replication *via* reverse transcription in the viral life cycle. Many studies have demonstrated that siRNA targeting different regions of the HBV genome can block viral replication and antigen expression[15-19]. Our previous study showed that siRNA targeting HBV nuclear localization signal (NLS) can inhibit viral DNA synthesis and cccDNA amplification[20]. As HBV infection is a cellular infection involving multiple genes, we hypothesize that combination of siRNAs targeting different sequences along the HBV NLS mediates a greater inhibitory efficacy on cccDNA amplication. If this is the case, it would be of significance in the treatment of HBV infection. In this study, we analyzed the kinetics of HBV genome replication during siRNA therapy and tested the antiviral capacity of combination therapy in comparison to monotherapy in HepG2.2.15 cells, showing that combination of siRNAs exhibits better effects on the inhibition of HBV replication, especially at viral cccDNA level.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM) and G418 were purchased from GIBCO BRL (USA). HepG2.2.15 cells were maintained in our laboratory. Plasmid psi/U6 was supplied by Wuhan JS Biotech (China). All polymerase chain reaction (PCR) primers were synthesized by Shanghai Boya Biological Company (China). Trizol, M-MLV reverse transcriptase, Lipofection 2000 reagent were purchased from Invitrogen Company (USA). Restriction enzymes were purchased from New England Bio-laboratory (Beijing, China).

Construction of siRNA express vector

siRNA expression plasmid was generated as previously described[21]. Briefly, 21-nucleotide long inverted sequences were cloned into the plasmid pGenesil/U6. Five thymidines were inserted into the downstream anti-sense strand to provide a stop signal for the polymerase Ⅲ RNA polymerase. The sense strand of hairpin was homologous to the target mRNA (NLS region) as analyzed by BLAST in the NCBI database. Oligonucletides used to code for the sense strand of siRNA included siRNA1 (5'-AAG ATCTCAATCTCGGGAATC-3'), siRNA2 (5'-CAGGT CCCCTAGAAGAAGAAC-3'), and control siRNAHK (5'-ACTACCGTTGTTATAGGTG-3'). All the plasmids constructed were confirmed by endonuclease digestion and DNA sequencing.

Cell culture and transfection

The human hepatoma cell line, HepG2.2.15, was maintained in Dubecco's modified Eagle's medium (DMEM) with 10% fatal bovine serum and 200 μg/mL G418 in 5% CO_2-humidified air as previously described[22]. Cells were cultur at a density of 3×10^5 cells per well in 6-well plates. Twenty-four hours after incubation, the cells were transfected with 4 μg siRNA-expressed plasmid using the Lipofection 2000 reagent. We removed the medium, washed the cells with warmed PBS, and added fresh medium every 24 h. At 48, 72 and 96 h after transfection, culture media were collected and cells were harvested for HBV replication assay. All experiments were performed in triplicate and divided into five groups.

Detection of HBsAg and HBeAg

To assess the effects of RNAi on viral antigen expression, HBsAg and HBeAg levels in culture medium were measured with an enzyme-linked immunosorbent assay (ELISA) kit following its manufacture's instructions.

Assay of HBV mRNA

Antiviral activities were evaluated by reverse-transcript PCR (RT-PCR). Total RNA was extracted directly from the transfected cells using Trizol reagent. Then, RNA was denatured for 5 min at 70℃, immediately cooled in ice water, reverse transcribed using M-MLV reverse transcriptase. A RT-PCR experiment targeting β-actin gene was run as an internal control. The primer sequences used are HBV forward (5'-ACCTC TGCCTAATCATCTC-3') and reverse (5'-GTAAG ACAGGAAATGTGAAAC-3'), β-actin forward (5'-GTCGGTGTGAACGGATTT-3') and reverse (5'-ACTCCACGACGTACTCAGC-3'). The PCR

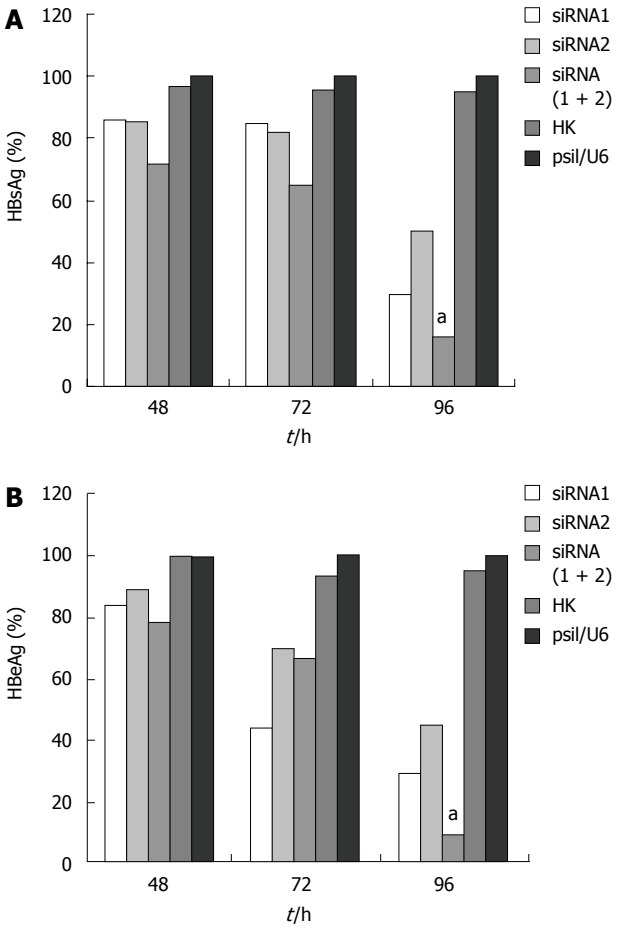

Figure 1 Relative expression of HBsAg (**A**) and HBeAg (**B**). $^{a}P < 0.05$ *vs* siRNA1 or siRNA2.

amplification product was analyzed by 1.2% agarose gel electrophoresis.

Quantitative real-time PCR

Real-time PCR was performed to quantify the HBV DNA or cccDNA using a HBV fluorescence detection kit following its manufacturer's protocol. For measurement of viral DNA, DNA was extracted from the culture supernatant using a QIAamp DNA mini kit. cccDNA was isolated from the transfection cells to quantify its level and examined every 24 h post-transfection according to its manufacture's protocol. Reactions with no reverse transcriptase enzyme added were performed in parallel. The inhibition ratio of HBV DNA was calculated according to the formula: 1-log (treated sample fluorescent intensity)/log (control fluorescent intensity) × 100%.

Dose-dependent inhibitory effect of single siRNA

To evaluate the dose-dependent effects of siRNA on HBV gene expression, a series of experiments were conducted in HepG2.2.15 cells transfected with different plasmids at the concentration of 2 μg, 3 μg and 4 μg, respectively. The transfection cells were harvested and cell culture supernatant was collected 96 h post-transfection for further examination. Several parameters

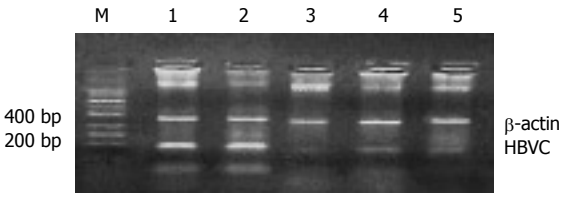

Figure 2 Reduction in HBV mRNA level after treatment with siRNA. M: marker, lane 1: HK, lane 2: psil/U6, lane 3: siRNAs 1 and 2, lane 4: siRNA2, lane 5: siRNA1.

of HBV were measured including HBsAg, HBV DNA as described above.

Statistical analysis

Statistical analysis was performed using the SPSS 12.0 software. The results were expressed as mean ± SD and compared using *F*-test and one-way ANOVA. $P < 0.05$ was considered statistically significant.

RESULTS

Inhibition of the expression of HBsAg and HBeAg

To test whether siRNA could effectively inhibit the expression of viral proteins in HepG2.2.15 cells, HBsAg and HBeAg in culture media were detected by ELISA. For HBsAg, cells transfected with the control siRNA produced an equal secretion with those treated with empty vector ($P < 0.05$) as expected in the absence of any specific silencing response, whereas cells expressing HBV siRNA gave a very different result. The HBsAg level in all the cells integrated with three siRNAs was significantly reduced compared with those transfected with empty vector, and the greatest reduction rate was 83.89% in the combined therapy group 96 h post-transfection (Figure 1A). HBeAg was also reduced in the selected siRNA-transfected cells, and the greatest reduction rate was 91.07% in the combined therapy group 96 h post-transfection (Figure 1B). For both HBsAg and HBeAg, combination therapy for siRNAs was more potent than any individual therapy.

Reduction of HBV mRNA level

To determine whether viral mRNA is efficiently degraded by siRNA, RT-PCR was performed 48 h, 72 h and 96 h after transfection. The results demonstrated that the mRNA level of each tested siRNA was markedly reduced, whereas the empty plasmid had no effect on mRNA level (Figure 2). Combination therapy with all the siRNAs produced a stronger inhibition on viral transcripts compared with the therapy with one siRNA. The HepG2.2.15 cells treated with combined siRNAs for 96 h did not contain any detectable mRNA, whereas mRNA was abundant in cells treated with empty vector.

Reduction of copies of viral DNA and cccDNA

The effect of siRNA silencing on HBV DNA was investigated by quantitative real-time PCR, which can detect 10^4-10^8 copies of HBV DNA. The level of viral

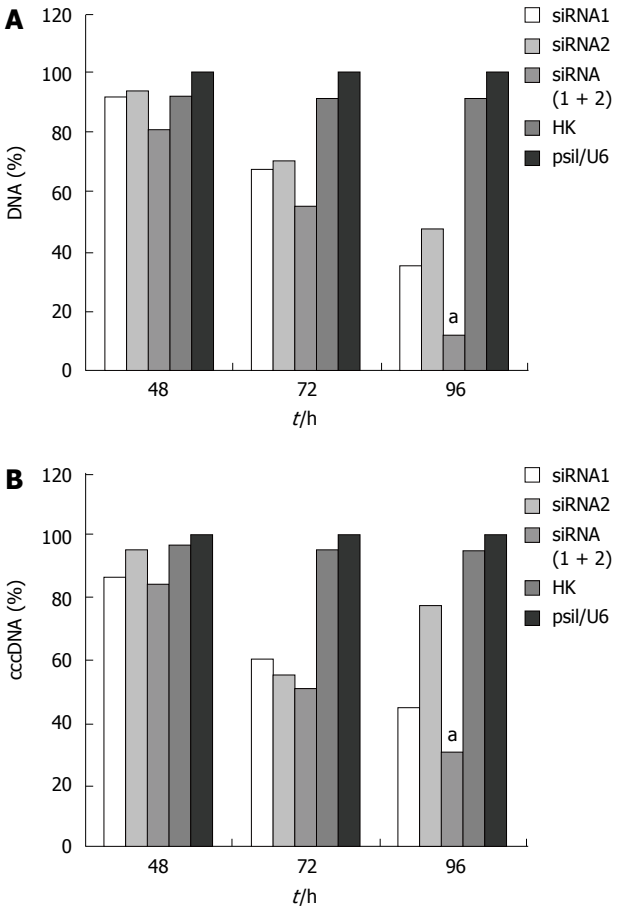

Figure 3 Inhibition of HBV DNA (**A**) and cccDNA (**B**) amplification after treatment with siRNA. [a]$P < 0.05$ *vs* siRNA1 or siRNA2.

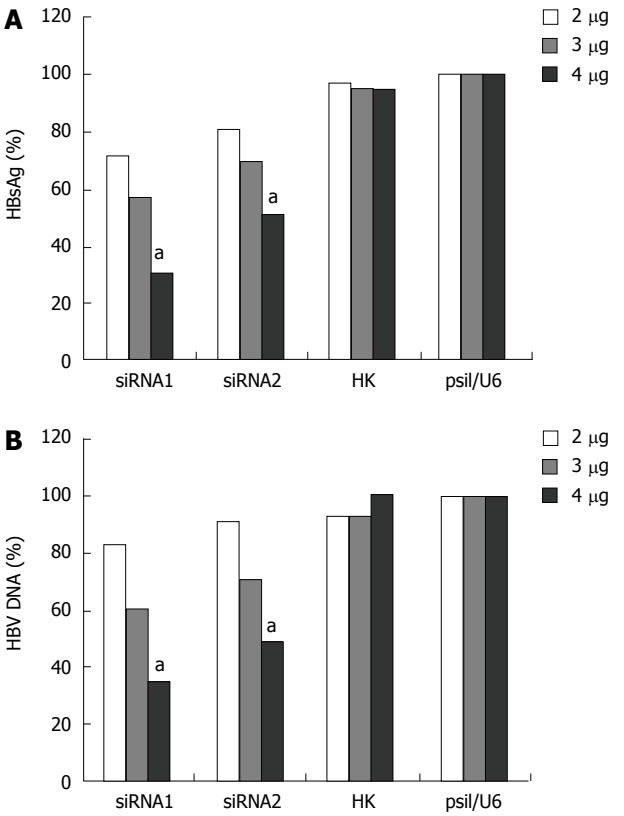

Figure 4 Dose-dependent inhibitory effects of siRNA on HBV antigen HBsAg expression (**A**) and HBV replication (**B**). [a]$P < 0.05$ *vs* 2 μg or 3 μg.

DNA in cells transfected with combined siRNAs was significantly lower than that in cells treated with empty vector. Treatment with irrelevant control siRNA slightly decreased the level of viral DNA in cells transfected with siRNA (Figure 3A). Furthermore, combined siRNA1 and siRNA2 showed a greater inhibitory effect (88.6%) on viral DNA replication than siRNA1 or siRNA2 alone ($P < 0.05$).

HBV cccDNA is an important parameter in the therapy for chronic HBV infection. To evaluate if combination of siRNAs can effectively inhibit viral cccDNA, we isolated viral cccDNA from the transfected cells every 24 h post-trasfection. Quantitative assay revealed that HBV cccDNA levels were decreased by 50.4%, 22.31%, and 69.83% in the cells transfected with siRNA1, siRNA2 and siRNA (1 + 2), respectively, compared to the level of cells treated with empty plasmid 96 h post-transfection. Meanwhile, HBV cccDNA levels did not significantly change in cells treated with control siRNA and empty vector. As expected, the use of siRNA1 and siRNA2 synergistically suppressed the viral cccDNA activities compared with siRNA1 or siRNA2 alone ($P < 0.05$, Figure 3B). These results were reproducibly observed in three independent experiments.

Dose-dependent effect of siRNA

As shown in Figure 4, siRNA had an obvious dose-dependent effect on HBV replication and antigen expression 96 h post-transfection. When the cells were transfected with a low concentration of siRNA, siRNA had almost no effect on HBV replication and antigen expression. However, a more significant inhibition of HBsAg and DNA in the cells treated at the concentration of 4 μg was observed. The level of HBsAg was decreased by 29%, 43% or 70% after siRNA1 treatment, and 9%, 30% or 50% after siRNA2 treatment, respectively, at the concentrations of 2 μg, 3 μg, 4 μg (Figure 4A). The level of HBV DNA was reduced by 17%, 39% or 65% after siRNA1 treatment, and 9%, 29% or 51% after siRNA2 treatment, respectively, at the concentrations of 2 μg, 3 μg, 4 μg (Figure 4B).

DISCUSSION

Chronic HBV infection is one of the most serious diseases threatening human health. RNAi technology provides an alternative strategy to combat HBV infection[23,24]. It was reported that viral mutations could escape single synthetic siRNA treatments, such as HBV, HCV and HIV[25-27]. One strategy to address the problem is to generate multiple siRNA molecules that can target different sites on the viral genome. The other is to choose target sequences in the relatively conserved region. Recently, combination therapy has emerged as a new approach to the treatment of chronic HBV infection with the objective to decrease the viral load

to the lowest possible levels[28,29]. This is then followed by a continued chemotherapy with another nucleotide analog or IFN-α in order to eliminate the remained viral load. Following the same strategy, we also investigated the effect of combination of siRNAs in comparison to monotherapy in HepG2.2.15 cells. As HBV infection is a pathological process involving multiple genes, it will be ideal to develop a combination strategy for siRNAs that can knock down the expression of multiple pathogenic viral antigens as well as inhibit viral replication. During the progress of combination therapy, several siRNAs may block multiple sites in viral genome and make it difficult to repair immediately, thus achieving greater suppression on HBV infection than a single siRNA.

The HepG2.2.15 cell line, a derivative of human HepG2 hepatoma cells, has been used as an *in vitro* stable HBV-producing model[22]. The cell line can be transformed with a head-tail dimmer of HBV DNA. Using this method, we could simulate the natural condition in which cells can still stably produce mRNA, antigen and viral particles. Specific siRNA targeting different sites on HBV NLS region were transfected and monitored in HepG2.2.15cells. As a result, the load of HBsAg, HBeAg, and mRNA was inhibited to some extent. Meanwhile, real-time PCR revealed that the number of viral DNA and cccDNA copies was markedly decreased. This inhibition was highly selective, sequence-specific and dose-dependent since control siRNA had almost no inhibitory effect on the expression or replication of HBV.

The selected siRNAs showed marked anti-HBV effects. Surprisingly, combination of siRNAs, compared with a single siRNA, exerted a stronger inhibition on antigen expression and viral replication, even though the final concentration of siRNA used in the therapy was the same. More importantly, combination therapy significantly suppressed HBV cccDNA amplification.

In summary, combination of siRNAs targeting various regions of HBV NLS inhibits viral replication and suppresses cccDNA amplication and can be used in the treatment of HBV infection.

COMMENTS

Background

Hepatitis B virus (HBV) is a major cause for acute and chronic hepatitis in humans. The development of an effective therapy for HBV infection is still a challenge. As HBV infection is a cellular infection involving multiple genes, progress in RNA interference (RNAi) has shed slight on developing a new anti-HBV strategy.

Research frontiers

siRNA targeting HBV nuclear localization signal (NLS) can inhibit viral DNA synthesis and cccDNA amplification. In this study, the authors hypothesize that combination of siRNAs targeting different sequences along HBV NLS mediates greater inhibitory effects on cccDNA. If this is the case, it would be of significance in the treatment of HBV infection.

Innovations and breakthroughs

In this study, the selected siRNA showed marked anti-HBV effects. Surprisingly, combination of siRNAs, compared with a single siRNA, exerted a stronger inhibition on antigen expression and viral replication, even though the final concentration of siRNA used in the therapy was the same. More importantly, combination therapy significantly suppressed HBV cccDNA amplification.

Applications

Combination of siRNAs targeting various regions of HBV NLS not only inhibits viral replication, but also suppresses cccDNA amplification, indicating that it can be used in the treatment of HBV infection.

Peer review

The effect of combination of siRNAs on suppressing HBsAg and HBeAg expression, and levels of HBV mRNA, DNA and cccDNA *in vitro* was studied in this study. This manuscript provides some important information about the treatment of chronic HBV infection. It is very interesting paper and has certain value.

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RAPID COMMUNICATION

Treatment of *Helicobacter pylori* in surgical practice: A randomised trial of triple *versus* quadruple therapy in a rural district general hospital

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Abstract

AIM: To compare a lansoprazole-based triple *versus* quadruple therapy for *Helicobacter pylori* (*H pylori*) eradication with emphasis on side effect profile, patient compliance and eradication rate at a rural district general hospital in Wales, United Kingdom.

METHODS: One hundred one patients with *H pylori* infection were included in the study. Patients were randomised to receive triple therapy comprising of lansoprazole 30 mg, amoxycillin 1 g, clarithromycin 500 mg, all *b.d.* (LAC), or quadruple therapy comprising of lansoprazole 30 mg *b.d.*, metronidazole 500 mg *t.d.s.*, bismuth subcitrate 240 mg *b.d.*, and tetracycline chloride 500 mg *q.d.s.* (LMBT). Cure was defined as a negative ^{13}C urea breath test 2 mo after treatment.

RESULTS: Seven patients were withdrawn after randomisation. Fifty patients were assigned to LAC group and 44 to LMBT group. The intention-to-treat cure rates were 92% and 91%, whereas the per-protocol cure rates were 92% and 97%, respectively. Side effects were common, with 56% experiencing moderate to severe symptoms in the LAC group and 59% in the LMBT group. Symptoms of vomiting, diarrhoea and black stools were significantly more common in the LMBT group. Patient compliance was 100% for triple therapy and 86% for quadruple therapy ($P < 0.01$). One-third of patients in both groups were still taking acid-reducing medications at six-month follow-up.

CONCLUSION: One-week triple and quadruple therapies have similar intention-to-treat eradication rates. Certain side effects are more common with quadruple therapy, which can compromise patient compliance. Patient education or modifications to the regimen are alternative options to improve compliance of the quadruple regimen.

Key words: *Helicobacter pylori*; Triple therapy; Quadruple therapy; Side effects; Treatment compliance; Eradication rate

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INTRODUCTION

European studies have shown that quadruple therapy, even though more effective with a cure rate of over 95% by per protocol analysis[1-3], is less popular compared to a standard triple therapy for eradication of *Helicobacter pylori* (*H pylori*) infection. The reasons for this are the complexity of the regimen and also its side effects. Scheduling drugs four or more times a day reduces compliance[4,5]. However, some studies have suggested that quadruple therapy has a similar magnitude of

adherence and adverse effects compared to triple therapies[6,7].

Triple therapies are the mainstay of current treatment but resistance to clarithromycin is reducing its effectiveness. In the presence of resistance to clarithromycin, some studies have shown eradication rate below 80% and even as low as 25%-61% with standard triple therapy containing clarithromycin, amoxycillin and a proton-pump inhibitor[7-11]. Clarithromycin resistance is also increasing in our region[12,13].

Quadruple therapy is used mainly as a second-line therapy after failed eradication with triple therapy[14-18]. Earlier consensus meeting reports including the Maastricht Ⅱ Consensus Report on the management of *H pylori* infection have recommended the use of quadruple therapy for 1 wk as second-line therapy for *H pylori* infection[19-21]. However, updated reports have now recommended quadruple therapy as an alternative first-line eradication therapy[22-24].

The objective of the study was to compare a standard lansoprazole-based triple therapy (HeliClear®) to a lansoprazole-based quadruple therapy as first-line therapy in a surgical practice in a predominantly Caucasian population in North Wales.

MATERIALS AND METHODS

We conducted a prospective randomised trial of patients under the care of an upper gastrointestinal surgeon at Ysbyty Gwynedd, a rural District General Hospital in North Wales. The population served by Ysbyty Gwynedd is predominantly (98.8%) white and there are about 120 new cases of *H pylori* each year from a population of around 180 000. Twenty-four percent of strains were resistant to metronidazole, 7% to clarithromycin and 4% to both. There was resistance to tetracycline in 1 out of 363 isolates and none to amoxycillin[12].

The Local Ethics Committee of the participating hospitals approved the study. From June 2001 to November 2005, 101 patients with diagnosis of *H pylori* infection proven by gastric histology or urease test or culture were included in the study. Two positive tests were required for inclusion. The inclusion and exclusion criteria are shown in Table 1.

Patients were recruited into the trial once they had met the criteria and given fully informed written consent. Patients were recruited from the outpatient departments at one district general hospital and a satellite hospital served by the same team of doctors. The patients received a 7-d course of either a triple regimen (LAC) or a quadruple regimen (LMBT) (Table 2).

Randomisation took place at the hospital pharmacies when the patients collected their medications with a note from the recruiting doctor. The pharmacists dispensed the medications adhering to the order on a random list of therapy regimens.

A printed chart showing the names of the drugs, the number of pills to take and the time schedule was given to all participants to improve understanding and compliance with treatment.

Table 1 Inclusion criteria and exclusion criteria

	Criteria
Inclusion criteria	Dyspeptic symptoms
	Has recent OGD (duodenal ulcer; gastric ulcer; gastritis or non-ulcer dyspepsia)
	Positive for *H pylori* on histology and culture or CLO test or ^{13}C-urea breath test
Exclusion criteria	Age less than 18 or above 75 yr
	Symptomatic gallstones
	Treated with antibiotic or bismuth-containing drugs during the month prior to inclusion
	Treated with proton pump inhibitor during the week prior to inclusion
	Disturbed gastrointestinal physiology (gastric surgery; vagotomy; Zollinger-Ellison syndrome; chronic ingestion of NSAIDs)
	Concomitant serious disease
	Concomitant medications that may adversely interact with the study drugs (e.g. warfarin, anti-epileptics)
	Pregnancy and breast-feeding
	Childbearing age without adequate contraception
	Allergy to drugs used in the study
	Mental illness
	Heavy drinking or abuse of drugs

Table 2 Regimens used in the trial

Triple therapy regimen (LAC)	Quadruple therapy regimen (LMBT)
Lansoprazole (30 mg *b.d.*)	Lansoprazole (30 mg *b.d.*)
Amoxycillin (1 g *b.d.*)	Metronidazole (400 mg *t.d.s.*)
Clarithromycin (500 mg *b.d.*)	Bismuth subcitrate (240 mg *b.d.*)
	Tetracycline chloride (500 mg *q.d.s.*)

Compliance was evaluated by patient's record of each dosage taken onto the chart during the week of therapy. Any tablet that was not consumed needed to be brought back to the clinic for pill count. The patients were asked to record the reasons for missed dosages. They were also asked to record any side effects and their severity during the therapy. Proton pump inhibitors and other acid-reducing medications were not allowed after treatment. The patients returned for interview at 6 wk after therapy. The efficacy of treatment was evaluated by means of the ^{13}C-urea breath test performed following the standard European protocol at 8 wk following the start of therapy[12]. Patients were reviewed again at 6 mo after therapy to assess symptoms and use of any medications after determining their post therapy *H pylori* status. Patients who tested positive were offered the alternate regimen and retested after a gap of 2 mo.

Statistical analysis

Proportions were compared using Fisher's Exact Test. Quantitative variables were compared using *t*-test and non-parametric variables were compared using Mann–Whitney *U* test. Non-categorical values are given as the mean ± SD. Calculations were performed using the SPSS for Windows statistical package.

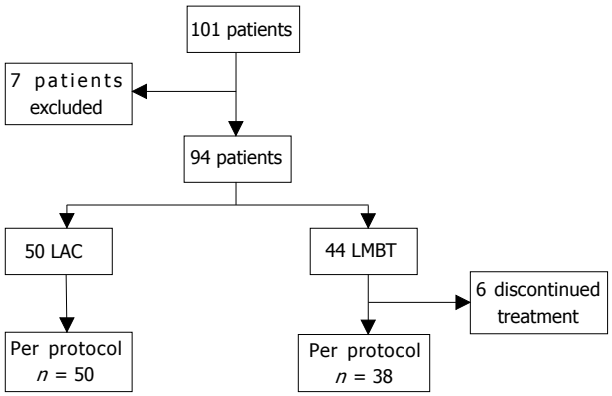

Figure 1 CONSORT flow diagram showing entries and withdrawals from the study.

Table 3 Patient characteristics

	Therapy	
	LAC (n = 50)	LMBT (n = 44)
Age	55.2 ± 10.9	53.7 ± 11.4
Gender (male: female)	26:24	27:17
Active smoking	10 (20%)	16 (36%)
NSAID use	4 (8%)	3 (7%)
Ethanol abuse (> 14 U/wk)	4 (8%)	3 (7%)
Previous therapy with antacids	4 (8%)	8 (18%)
Time between treat-ment and UBT (mo)	2.2 ± 0.7	2.1 ± 0.5
Gastric ulcer	1	1
Duodenal ulcer	3	1
Gastritis	36	33
Duodenitis	6	8
Diagnosis of *H pylori* infection (Urease: Culture: Biopsy)	42:29:45	37:27:44

NSAID: Non-steroidal anti-inflammatory drug; UBT: Urea breath test.

RESULTS

One hundred one patients were randomized into the trial but seven patients were withdrawn from the study after randomization (one because of diagnosis of bronchial carcinoma, one because of diagnosis of gallstones, two withdrew from the study and three were non-compliant to study protocol) (Figure 1).

Fifty patients were assigned to the LAC group and 44 to the LMBT group. The demographic and clinical characteristics of the groups were comparable (Table 3).

Compliance and side-effects

Compliance was excellent in the LAC group with all the patients completing the 7-d therapy. In contrast, 6 patients (14%) in the LMBT group failed to complete the treatment ($P < 0.01$). In spite of this three had a negative breath test.

Four out of the six patients had attributed moderate/severe nausea as the reason for discontinuing treatment. One had severe diarrhoea and another had nausea, vomiting and diarrhoea.

Side effects were reported by vast majority of patients in both groups, 45 patients (90%) in the LAC group and 42 patients (95%) in the LMBT group.

Table 4 Incidence of side effects n (%)

	Therapy		*P*-value
	LAC (n = 50)	LMBT (n = 44)	
Nausea	11 (22)	20 (45)	< 0.05
Vomiting	0 (0)	9 (20)	< 0.01
Diarrhoea	14 (28)	25 (57)	< 0.01
Headache	12 (24)	19 (44)	
Dizziness	9 (18)	11 (25)	
Blurred vision	5 (10)	6 (14)	
Itching	5 (10)	5 (11)	
Rash	1 (2)	2 (5)	
Dry mouth	27 (54)	19 (43)	
Sore mouth	4 (8)	0 (0)	
Glossitis	2 (4)	1 (2)	
Black tongue	6 (12)	6 (14)	
Black stool	5 (10)	35 (80)	< 0.01
Taste disturbance	23 (46)	14 (32)	
Arthralgia	3 (6)	1 (2)	

Table 5 Severity of side-effects (n)

	Therapy	
Severity	LAC	LMBT
None	5	2
Mild	17	19
Moderate	25	13
Severe	3	10
Total	50	44

Severity score: 1 = mild, does not cause any concern; 2 = moderate, but not bad enough to discontinue treatment; 3 = severe or incapacitating, forced to discontinue treatment.

The most frequent symptoms in the LAC group were dry mouth (54%) and taste disturbance (46%). Patients in the LMBT group experienced significantly more nausea (45%), vomiting (20%), diarrhoea (57%) and black stool (80%) (Table 4).

Each symptom was graded as mild, moderate or severe. In the LAC group, mild symptoms were observed in 17 patients (34%), moderate symptoms observed in 25 patients (50%) and severe symptoms observed in 3 patients (6%). In the LMBT group, mild symptoms were observed in 19 patients (43%), moderate symptoms observed in 13 patients (30%) and severe symptoms observed in 10 patients (23%) (Table 5, $P < 0.05$). Despite most of the patients experiencing some side effects, none were severe enough to require hospitalization.

^{13}C-urea breath test

All 94 patients returned for a ^{13}C-urea breath test 2 mo after eradication therapy. Four patients (8%) from the LAC group and four patients from LMBT (9%) had positive results indicating failure of *H pylori* eradication. Three of the four patients had an incomplete quadruple therapy (Table 6).

All the eight patients who tested positive with ^{13}C-urea breath test had the alternate regimen. Three of four patients, who had initially LAC and then LMBT therapy, were negative on the second breath test.

Table 6 ^{13}C-urea breath test results *n* (%)

	Therapy		*P*-value
	LAC (*n* = 50)	LMBT (*n* = 44)	
Returned for UBT	50 (100)	44 (100)	-
Completed therapy	50 (100)	38 (86)	< 0.01
UBT result	46 negative, 4 positive	37 negative, 1 positive	
Not completed therapy	0 (0%)	6 (14)	< 0.01
UBT result	-	3 positive, 3 negative	-
Intention-to-treat cure rate	92% (46/50)	91% (40/44)	
Per-protocol cure rate	92% (46/50)	97%(37/38)	

UBT: Urea breath test.

Table 7 Symptomatic outcome at 6-mo follow-up *n* (%)

	Therapy	
	LAC (*n* = 50)	LBMT (*n* = 44)
Follow-up at 6 mo	46 (92)	40 (91)
Persistent symptoms	4 (8)	7 (16)
Recurrent symptoms	17 (34)	9 (20)
Repeat eradication therapy	1 (2)	1 (2)
Long-term acid-reduction therapy	17 (34)	14 (32)

Six-month follow-up

Eighty-six patients (91.5%) returned for a 6-mo follow-up. Over one-third of patients had recurrent or persistent symptoms and remained on long-term acid-reduction therapy (with proton-pump inhibitors, H_2-antagonist or other antacids) even after successful eradication (Table 7).

DISCUSSION

This study has shown that a lansoprazole–based quadruple therapy is as effective as triple therapy in a predominantly white population in the UK (intention-to-treat rate: 91% *vs* 92% respectively). The resistance to clarithromycin (7%) is beginning to diminish the effectiveness of the triple therapy (92% per protocol eradication) whereas metronidazole resistance (24%) did not affect quadruple therapy (97% per protocol eradication)[25].

Side effects are common in both regimens occurring in around 90% of patients. However, severe side effects occurred more frequently with quadruple therapy (23% *vs* 6%) and this reduced compliance.

Four out of the six patients taking quadruple therapy stopped because of nausea and vomiting, which was probably due to metronidazole. Replacing metronidazole with amoxycillin should reduce these side effects and increase compliance[26]. Interestingly, dry mouth was noticed more in the triple therapy group even though lansoprazole was the most likely cause.

The intention-to-treat cure rate of quadruple therapy (LMBT) was comparable to triple therapy (LAC) in spite of lower compliance. Educating patients about the possible common side effects and the importance of complete eradication should provide a very high cure rate as the per protocol cure rate was 97% for quadruple therapy.

Quadruple therapy is very cost effective and should be considered as a first-line therapy especially when there are economic constraints. Lansoprazole-based quadruple therapy costs £17 as against £38 for the triple therapy for a one-week course[27]. The difference of £21 per treatment can be relieved from economic burden for the health service to treat this common condition.

Patients have to be warned that about one sixth of them will have persistent symptoms and about third of them will develop recurrent symptoms with a similar proportion needing long-term treatment with a proton-pump inhibitor, H_2-antagonist or other antacids.

Modified seven-day quadruple therapy, by reducing the frequency of tetracycline chloride and bismuth subcitrate from four times to three times daily, has also been tried successfully as a first-line treatment with cure rate and compliance rate of over 90%[2]. Bateson has shown that a twice-daily quadruple therapy using lansoprazole, tetracycline, clarithromycin and metronidazole is effective (95.5% eradication rate) in UK patients with duodenal ulcer but this pre-dated resistance to clarithromycin and metronidazole[28]. Amoxycillin has been shown to improve eradication in resistant patients and perhaps a trial of a twice-daily quadruple therapy substituting amoxycillin for metronidazole should be considered[26]. Other approaches to the problem of antibiotic resistance include a sequential therapy that substituted amoxycillin with tinidazole during the first 5 d of a 10-d triple therapy with pantoprazole, amoxycillin and clarithromycin, which has been shown to achieve a significantly higher eradication rate[29]. Pretreatment sensitivity testing has been confirmed to be cost effective by significantly improved eradication in a study that used omeprazole and two antibiotics chosen based on susceptibility testing, compared to omeprazole, clarithromycin and metronidazole standard triple therapy[30].

Recent randomised studies that compared triple therapy with quadruple therapy as a first-line treatment option for *H pylori* and some reports showed superior eradication rates with the quadruple therapy[6,31,32] whereas others have shown no difference[33,34]. Quadruple therapy is becoming the standard treatment as resistance to clarithromycin, and to a lesser extent metronidazole, is reducing the efficacy of triple therapies. The side effects may be reduced by replacing metronidazole with amoxycillin but patients should be better educated about the side effects in order to improve compliance and cure rates.

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COMMENTS

Background

The treatment for *Helicobacter pylori* (*H pylori*) is becoming less effective as the organism is becoming resistant to the commonly used antibiotics in triple

therapies. Quadruple therapies were less popular because of their side effects but still have good eradication rates.

Research frontiers

This study compares lansoprazole-based triple and quadruple therapy for *H pylori* infection in white Caucasians in rural Wales, an area with low resistance to Clarithromycin and moderate resistance to metronidazole.

Innovations and breakthroughs

Both regimens had high eradications rates (> 90%) showing that resistance has not yet significantly affected this UK population. Even better rates (97%) can be achieved with quadruple therapy if patients are able to complete the full course. Patients need to be educated about the side effects and importance of completing the course to achieve the higher eradication rates.

Applications

Quadruple therapies provide a cost effective and highly successful treatment for *H pylori*. The side effects and compliance may be improved by substituting amoxycillin for metronidazole-an area for future research.

Terminology

Triple therapy is a regimen of a proton pump inhibitor and two antibiotics. Quadruple therapy is a regimen of a proton pump inhibitor, a bismuth compound and two antibiotics.

Peer review

The authors compared lansoprazole-based triple and quadruple therapy in the eradication of *H pylori*. They found that both regimens were equally effective and that quadruple therapy was less costly even though 6 patients had to discontinue treatment because of side effects. This is an important study.

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RAPID COMMUNICATION

Pathological evolution of hepatitis C virus-"Healthy carriers"

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Abstract

AIM: To determine factors associated with fibrosis progression in hepatitis C virus (HCV)-infected patients without significant initial pathological lesions.

METHODS: Seventy six untreated HCV-infected patients with initially normal liver as defined by a Knodell score $\leqslant$ 3, with 2 liver biopsies and detectable HCV-RNA were included. Markers of fibrosis progression were assessed.

RESULTS: Median duration of infection and time between paired biopsies was 13 (95% CI: 1-28) and 4 (95% CI: 2-16) years respectively. Alanine-transaminase (ALT) activity was normal in 43.4% of cases. 50% demonstrated progression of the necro-inflammation and 34% of fibrosis after a median time evolution of 4 years (95% CI: 2-16). The median difference in the necro-inflammation and fibrosis score between biopsies was low, 1.5 and 0.0 respectively. Univariate analysis showed there was no difference between fibrosis activity or evolution according to genotype or viral load. A higher fibrosis progression (P = 0.03) was observed in patients with body mass index (BMI) > 25. Fibrosis progression correlated with the time interval between biopsies (P = 0.01). A significant progression of activity (1.7 *vs* 0.4, P < 0.05) or fibrosis (0.9 *vs* 0.0, P < 0.01) was observed in patients with elevated ALT. There was a significant correlation between activity progression and fibrosis progression (P = 0.003). Multivariate analysis demonstrated that fibrosis progression was associated with elevated ALT, BMI > 25 and the time interval between 2 biopsies.

CONCLUSION: There is no fibrosis progression in 66% of patients without significant initial histopathological lesion. Fibrosis progression is associated with elevated ALT and BMI > 25.

Key words: Hepatitis C virus; Liver fibrosis; Liver biopsy; Alanine-transaminase; Body mass index

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INTRODUCTION

Several studies have evaluated the long-term outcome of fibrosis progression in patients with chronic active hepatitis. A normal liver is observed in about 10% of infected patients as defined by the absence of significant pathological changes[1-3]. The natural history of these so-called "healthy hepatitis C virus (HCV) carriers" is not fully defined. Studies usually differentiate patients with or without alanine-transaminase (ALT) abnormalities but rarely patients with or without liver biopsy abnormalities[4]. It is estimated that 10% to 40% of HCV-infected patients have a carrier state with normal ALT and a slower natural course of the disease than patients with elevated ALT[5-9]. The aim of this study was to better define the pathological spontaneous evolution in HCV-infected immunocompetent patients without specific histopathological lesions.

MATERIALS AND METHODS

Among our file of 3600 HCV-infected patients in the

liver unit, we selected retrospectively those fulfilling the following criteria: detectable HCV RNA, Knodell score ⩽ 3 at the first pathological evaluation[10], at least 2 sequential liver biopsies in the absence of antiviral therapy or HIV and HBV confection. Kidney transplant recipients or hemodialyzed patients were excluded as well as patients with other causes of chronic liver disease (hepatotoxic drugs, autoimmune chronic hepatitis, hemochromatosis, Wilson's disease and alpha 1 antitrypsin deficiency). In our center we usually perform a liver biopsy in all HCV-RNA chronic carriers, whatever the transaminase levels. In the case of low pathological lesions, we usually propose therapeutic abstention, a biochemical follow-up twice a year, an abdominal ultrasonography (US) yearly and a pathological follow-up with a liver biopsy every three to five years. In patients infected by blood transfusion or intravenous drug use, duration of HCV infection was estimated as the time elapsed from the year of transfusion or intravenous drug use (IVDU) onset to that of the first liver biopsy. Gender, route of infection, HCV viral genotype and serum viral load, body mass index (BMI), alcohol consumption before the first biopsy, serum ALT and serum glutamyltransferase (GGT) levels were recorded for each patient.

Histological analysis

Liver biopsy specimens were fixed, paraffin-embedded, and routinely stained with haematoxylin-eosin and Masson's trichrome and picrosirius red for collagen. For each liver biopsy specimen, stage of fibrosis (from 0 to 4) and grade of necro-inflammation including portal inflammation (from 0 to 4), periportal piecemeal necrosis (from 0 to 10) and intralobular inflammation (from 0 to 4) were established according to the Knodell score criteria. Worsening of the necro-inflammation (sum of portal inflammation, periportal piecemeal necrosis and intralobular inflammation scores) and fibrosis were defined by an increase of at least 2 and 1 points, respectively.

RNA quantification and procedure for HCV genotyping

Serum HCV RNA quantitative detection was performed using the RT-PCR method with a sensitivity limit of 100 copies/mL (Amplicor® Roche, Switzerland). Genotypes were identified using the INNO-LIPA HCV procedure (Innogenetics, Belgium).

Statistical analysis

SPSS software version 10.0 (SPSS Inc, Chicago, Illinois, USA) was used for statistical analysis. Quantitative variables were compared using Student's *t*-test or nonparametric Mann-Whitney variance analysis (ANOVA). Qualitative variables were compared using the χ^2 test or the Fischer test when necessary. Multivariate analysis was done using robust logistic regression. A two-tailed *P* value less than 0.05 was considered as significant.

RESULTS

Among our HCV-infected patient group, 410 patients had a first liver biopsy with a Knodell score ⩽ 3. Only 76 patients, 34 males and 42 females, had at least a second liver biopsy and fulfilled the selection criteria. Their main characteristics are given in Table 1. Mean age at infection and at the first biopsy were 25 ± 9 years and 38 ± 9 years, respectively. Thirty-three patients (43.4%) had been contaminated by intravenous drug use and 28 (36.8%) by transfusions. Forty patients (55.6%) were infected with genotype 1 and 15 (20.8%) with genotype 3. The median duration of infection before the first biopsy was 13 (95% CI: 1-28) years and the median time between paired biopsies was 4 (95% CI: 2-16) years. The mean number of ALT level available between the 2 biopsies was 6 ± 3. During the follow-up period, 33 patients (43.4%) had normal serum ALT activity, 33 (43.4%) patients displayed occasional mild ALT increases (less than 2 times the upper normal limit) and 10 patients (13.2%) had constantly elevated ALT (> 3N). The mean daily alcohol consumption was 22.2 g/d (95% CI: 0-250). Mean BMI was 23.5 ± 3.1 for males and 22.0 ± 5.1 for females, without significant difference. Forty patients (55.6%) were infected with genotype 1; 9 (12.5%) with genotype 2; 15 (20.8%) with genotype 3; 6 (8.3%) with genotype 4 and 2 (2.8%) with genotype 5. Genotype was unknown for 4 patients. Viral load was low (under 350 000 UI/mL) for 33 patients (55%), medium (350 000 to 700 000 UI/mL) for 14 patients (23.3%) and high (more than 700 000 UI/mL) for 13 patients (21.7%). At the first biopsy, mean values for necro-inflammation and fibrosis were 1.75 ± 0.68 and 0.57 ± 0.5 respectively.

At the last biopsy, a significant increase in necro-inflammation (⩾ 2 points) and fibrosis score (⩾ 1 point) was observed in 38 (50%) and 26 patients (34%) respectively; 3 patients having a fibrosis equal to 3 and one equal to 4 (Figure 1). The mean difference in the necro-

Table 1 Demographic and clinical features of HCV patients with initially normal liver

Demographic and clinical features	Data
Number of patients	76
Sex (M/F)	34/42
Age at the first biopsy (mean, yr)	38 ± 9
Age at Infection (mean, yr)	25 ± 9
BMI (M/F)	23.5 ± 3.1/22 ± 5.1
Route of infection:Transfusion	28 (36.8%)
IVDU	33 (43.4%)
Other or unknown	15 (20%)
Genotype 1/2/3/4/5	40/9/15/6/2
Infection duration, median (95% CI)	13 (1-28)
Times between 2 biopsies, median (95% CI)	4 (2-16)
Alcohol consumption (g/d, mean ± SD)	22.2 ± 44
ALT level between 2 biopsies	
Constantly normal	42 (55.3%)
Normal or < 2 N	31 (40.8%)
> 2 N	3 (3.9%)
Viral load:	
Low (under 350 000 UI/mL)	33 (55%)
Medium	14 (23.3%)
High (more than 700000 UI/mL)	13 (21.7%)
Knodell score at the first liver biopsy	
Necroinflammatory index (mean ± SD)	1.7 ± 0.7
Fibrosis score (mean ± SD)	0.6 ± 0.5

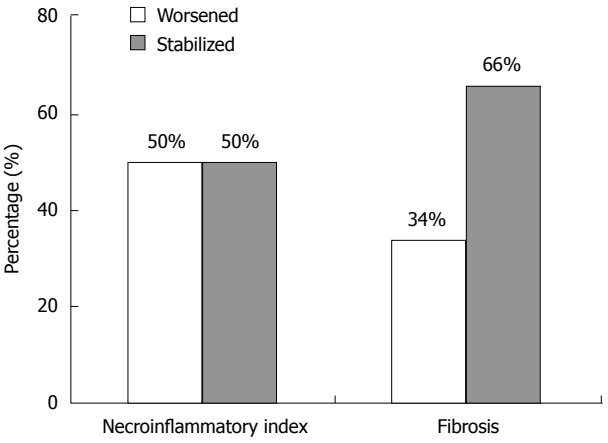

Figure 1 Pathological evolution. 38 patients (50%) had progression of necroinflammatory activity (progression ⩾ 2) and 26 (34%) had progression of fibrosis (progression ⩾ 1; 3 patients having a fibrosis score at 3 and one at 4).

inflammation and fibrosis scores between the 2 biopsies was low: 1.79 ± 2.23 and 0.42 ± 0.77, respectively. Univariate analysis showed there was no difference between activity and fibrosis evolution according to the genotype, viral load or the infection duration. A higher fibrosis progression (1.00 ± 1.3) was observed in patients with BMI > 25 as compared to patients with BMI < 25 (0.28 ± 0.53) ($P = 0.03$). A significant progression in activity (mean = 1.7 ± 0.8 *vs* 0.4 ± 0.5) ($P < 0.05$) or fibrosis (mean = 0.9 ± 0.3 *vs* 0.0 ± 0.2) ($P < 0.01$) was observed in patients with elevated ALT as compared to patients with normal ALT. There was also a significant correlation between activity progression and fibrosis progression ($P = 0.003$).

By multivariate analysis, factors independently associated with liver fibrosis progression were an elevated ALT (RR = 7.5, CI = 1.4) ($P = 0.02$), BMI > 25 (RR = 4.9, CI = 1.2) ($P = 0.03$) and the interval between the 2 biopsies (RR = 1.8, CI = 1.3) ($P = 0.001$).

DISCUSSION

The long-term natural history of the so-called healthy carriers of HCV is not clear. Although ALT levels do not reflect the severity of the liver damage [11], patients with persistently normal ALT levels usually have a less severe disease, corresponding to a lower progression of fibrosis[8,12-15]. Nevertheless, some reports suggest the presence of significant fibrosis or cirrhosis in some of them[8,13,16,17]. Other reports underline the relationship between excess weight and hepatitis C-related fibrosis progression[18,19]. This study was designed to evaluate whether HCV-infected subjects with pathologically normal liver had any progression of liver damage after 4 years of follow-up. This might allow the screening of patients for which antiviral treatment would be helpful.

In our study, fibrosis progression only concerns one third of the patients. The short time between two biopsies cannot exclude a later worsening of liver fibrosis, which is probably slow, with a low risk of evolution to cirrhosis. Although ALT levels do not reflect the severity of the liver disease, elevated ALT was associated with liver fibrosis progression in this population with initially normal liver. Overweight, accordingly to the recent literature was also associated with fibrosis worsening[19]. Patients with elevated ALT or BMI may have more necroinflammatory activity resulting in more pronounced fibrosis progression[19-21].

Our results suggest that ALT level follow-up is necessary whatever the histopathological results. In the subgroup of patients with elevated ALT or BMI, pathological follow-up seems to be useful and weight loss should be proposed. Our study suggests that liver fibrosis progression is correlated with time between biopsies, which probably make histopathological controls necessary. In patients with normal liver, normal ALT level and without co-morbidities such as excess weight, the time between biopsies should be longer than 5 years.

In our study, alcohol consumption was not correlated with fibrosis progression. We recorded the alcohol consumption before the first biopsy. We can suggest as a major hypothesis for this unusual result, that patients reduced their alcohol consumption after knowledge of their HCV status during follow up[22].

Antiviral therapy is usually not used in the so-called "asymptomatic HCV-carriers"[23,24]. We can differentiate in these "asymptomatic carriers" a sub-group which is at risk of fibrosis progression. The question is whether and when pegylated interferon and ribavirin should be a therapeutic option? Theoretically, if patients with normal liver tests do not really need treatment, therapy still can be proposed to interested patients with the same response rate as patients with elevated transaminases [25-29]. In addition, patients with a higher risk of liver fibrosis progression should be treated, particularly in case of genotype 2 or 3 infection[30].

In conclusion, this study confirms that the "HCV-healthy carrier" state does exist. Fibrosis does not worsen in two thirds of HCV-carriers without histopathological features after 4 years, supporting the concept that the natural history of chronic hepatitis in this group of subjects is characterized by a very slow or no progression. Antiviral therapy is not recommended in these patients with normal ALT or BMI under 25. Overweight, HCV-infected patient should be informed of the risk of liver fibrosis progression and the need of dietetic councils.

COMMENTS

Background

Chronic hepatitis C virus (HCV) infection cause liver damage, with a fibrotic scarring, which can progress to cirrhosis. The natural history of the infection varies among patients. For instance, in 10% of the case, the liver appears pathologically normal. Altogether, 10% to 40% of HCV infected patients harbor normal liver tests and the disease progresses very slowly as compared with patients with elevated liver tests. Until now, studies usually differentiate patients with or without liver test abnormalities but rarely patients with or without liver biopsy abnormalities.

Research frontiers

The goal of this work was to evaluate and differentiate patients for whom treatment will offer a better management of the disease. Among HCV infected

patients without significant liver damage, one-third progress toward fibrosis. This work focuses on early detection of these patients with a view to treatment before fibrosis onset.

Innovations and breakthroughs

In two thirds of HCV infected patients without significant liver damage, there is no fibrosis progression. In these patients, fibrosis progression is associated with abnormal liver tests and elevated BMI.

Applications

These observations may be helpful to suggest if a patient should receive an antiviral therapy. A treatment should be counselled to the patients with abnormal liver test and elevated BMI.

Terminology

Liver fibrosis is the excessive accumulation of a scarred tissue that occurs in most types of chronic liver diseases. This fibrosis can progress to cirrhosis. The transaminases are a group of liver enzymes including alanine aminotransferase (ALT). Elevated transaminases can be an indicator of liver damage.

Peer review

Sobesky *et al* present an interesting study describing the features and development of individuals with chronic hepatitis C infection and almost normal histological findings. The paper is properly written.

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RAPID COMMUNICATION

Nuclear β-catenin expression as a prognostic factor in advanced colorectal carcinoma

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Abstract

AIM: To investigate the changing pattern of β-catenin expression and its prognostic value in advanced colorectal cancer (CRC).

METHODS: Archival tumor samples were analyzed for β-catenin using immunohistochemistry (IHC) in 95 patients with advanced CRC.

RESULTS: Membranous β-catenin expression was found in the normal colorectal epithelium. Almost 100% of CRC cases showed membranous and cytoplasmic expression, and 55 (58%) cases showed nuclear expression. In univariate (Kaplan-Meier) survival analysis, only the nuclear index (NI) was a significant predictor of disease-free survival (DFS) (P = 0.023; n = 35), with a NI above the median associated with longer DFS (34.2 mo) than those with a NI below the median (15.5 mo) (P = 0.045, ANOVA). The other indices were not significant predictors of DFS, and none of the three tested indices (for membranous, cytoplasmic, or nuclear expression) predicted disease-specific survival (DSS). However, when dichotomized as positive or negative nuclear expression, the former was a significant predictor of more favorable DFS (P = 0.041) and DSS (P = 0.046).

CONCLUSION: Nuclear β-catenin expression provides additional information in predicting patient outcome in advanced CRC.

Key words: Colorectal carcinoma; β-catenin membrane staining; Cytoplasmic staining, Nuclear staining; Immunohistochemistry; Prognosis; Disease-free survival; Disease-specific survival

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INTRODUCTION

β-catenin is a 92-kDa multifunctional protein that, in its membrane location, links the intracellular part of the E-cadherin complex to actin cytoskeleton, which is a critical step in morphogenesis and maintenance of tissue integrity[1]. Alternatively, through Wnt signaling-mediated stabilization, β-catenin may act as a down-stream transcriptional trans-activator of several target genes[2]. Alterations in β-catenin protein expression levels and genetic rearrangement located in β-catenin exon 3 have been shown to contribute to the malignant character of various carcinomas and are likely to affect both intercellular adhesion and signal transduction, which are believed to be two independent functions of β-catenin protein[3].

The rare occurrence of mutations in β-catenin exon 3 has been previously documented in ulcerative colitis-related neoplastic progression[4] and in colorectal cancer (CRC) as well[5]. Immunohohistochemical studies suggest that the observed accumulation in β-catenin protein is probably due to genomic alterations in β-catenin coding regions, particularly in exon 3.

The impact of aberrations in β-catenin expression on the clinical outcome of CRC is controversial. Some studies reported prognostic value for cytoplasmic rather than nuclear expression[6], whereas others showed that nuclear accumulation of β-catenin may be an independent marker of unfavorable prognosis[7,8]. The aim of this study was to evaluate the possible role of β-catenin expression as a predictor of clinical outcome in advanced CRC patients.

MATERIALS AND METHODS

Study material

Ninety-five patients with advanced colorectal carcinoma (CRC), enrolled consecutively from CRC patients attending our clinic for therapeutic procedures during the late 1990s, were included in our study. Of these 95 patients, 60 had metastases at diagnosis (Stage Ⅳ disease), while the remaining 35 patients (with stage Ⅱ and Ⅲ disease at baseline) subsequently developed a metastatic disease during the mean follow-up (FU) time of 25.1 ± 27.8 (SD) mo. All patients were treated at the Department of Oncology and Radiotherapy, Turku University Hospital, according to the protocols in routine use for the treatment of CRC patients with stage Ⅱ, Ⅲ or IV disease at that time. The 95 patients included in the present study were enrolled into the study cohort between October 1998 and August 2003. All patients were prospectively followed-up until death or until their last clinical visit (March 2007), with the median FU-time of 27.6 (range 3-150) mo. The study was approved by the TUH Ethics Committee and was conducted in accordance with the Declaration of Helsinki. Samples were collected with the endorsement of the National Authority for Medico-legal Affairs.

Key clinical data for these patients are shown in Table 1. Of the 95 cases, 38 were women and 57 were men. The mean age was 61.5 (range 24-78) years. The majority (n = 39) of the tumors were localized in the left colon, followed in the order of frequency by the right colon (n = 24), rectum (n = 24), and colon transversum (n = 7). At the time of diagnosis, 15 patients were Stage Ⅱ, 20 were Stage Ⅲ and 60 patients were Stage Ⅳ. Accordingly, the majority (n = 63, 66.3%) had T3 tumors, and almost half of the patients had lymph node involvement at the time of diagnosis (n = 46). The patients were selected for the cohort on the basis of both the diagnosis and treatment they received, and each patient was assigned to one of two treatment arms: (1) 20 were treated with irinotecan alone, and (2) 75 received a combination of irinotecan and 5-fluorouracil (5-FU). The chemotherapy regimen the patients received was included in a previous study investigating irinotecan combined with bolus 5-fluorouracil and folinic acid[9].

Table 1 Key characteristics of patients and their tumors

Variable	No. or value	%[1]
Patients	95	
Male	57	60.0
Female	38	40.0
Age (yr)		
Median (range)	60.7 (24-80)	
Primary tumour status[2]	95	
T1	1	1.1
T2	6	6.3
T3	63	66.3
T4	17	17.9
Tx	8	8.5
Primary nodal status[2]	95	
N0	25	26.3
N+	46	48.4
Nx	24	25.3
Metastases at diagnosis[2]	95	
M0	35	36.8
M1	60	63.2
Histological grade	95	
Gr Ⅰ	12	12.6
Gr Ⅱ	62	65.3
Gr Ⅲ	18	19.0
NA	3	3.2
Stage	95	
Stage Ⅱ	15	15.8
Stage Ⅲ	20	21.0
Stage Ⅳ	60	63.2
Survival (mo)		
From primary diagnosis Median (range)	27.3 (3-150)	
From metastasis Median (range)	21.4 (3-80)	

[1]When applicable; [2]TNM classification; T_X: Unknown, Nx: Unknown, NA: Not available.

β-catenin immunostaining

Formalin-fixed, paraffin-embedded primary colorectal tumor tissue was obtained from 95 patients. Sections were cut serially at 5 μm for routine haematoxylin and eosin staining and for immunohistochemical (IHC) analysis. An experienced pathologist confirmed all histological diagnoses. IHC analysis was done using an automatic system (BenchMark XT, Ventana Medical Systems, Inc. Tucson, Arizona, USA). This fully automated processing of bar code labeled slides included baking of the slides, solvent free deparaffinization, antigen retrieval in a cell conditioning buffer CC2 (Mild: 36 min conditioning, and standard: 60 min conditioning), and incubation with the monoclonal mouse β-catenin antibody (clone CAT-5H10, isotype IgG1-kappa, Zymed Laboratories, San Franscisco, CA) at a dilution 1:200 (32 min, 37°C). The dilution of the primary antibody was based on previous dilution experiments. UltraViewTM Universal DAB (a biotin-free, Multimer-based detection system for the specific and sensitive detection of mouse IgG, mouse IgM, and rabbit IgG primary antibodies) was used. UltraView DAB includes: ultraView Universal HRP, ultraView Universal DAB Inhibitor, ultraView Universal DAB Chromogen, ultraView Universal DAB H_2O_2, and ultraView Universal DAB Copper. Counterstaining with haematoxylin (2021) was done for 4 min, and post-counterstaining with a blueing reagent (2037) was done for 4 min as well. After staining, the sections were dehydrated in ethanol, cleared in xylene, and covered with Mountex and cover slips.

Evaluation of β-catenin staining

β-catenin staining was evaluated using regular light microscopy by an observer who was blind to the clinical data (AB). All membranous, cytoplasmic, and nuclear staining were evaluated separately. For cell membrane staining, four categories were used (+++, ++, +, -), starting from equivalent to normal to entirely negative[10]. The cytoplasmic staining was also graded into four categories: (0) Negative, no detectable staining, (1) Weak, but still detectable staining, (2) Moderate, clearly positive but still weak, (3) Heavy staining, intense[11].

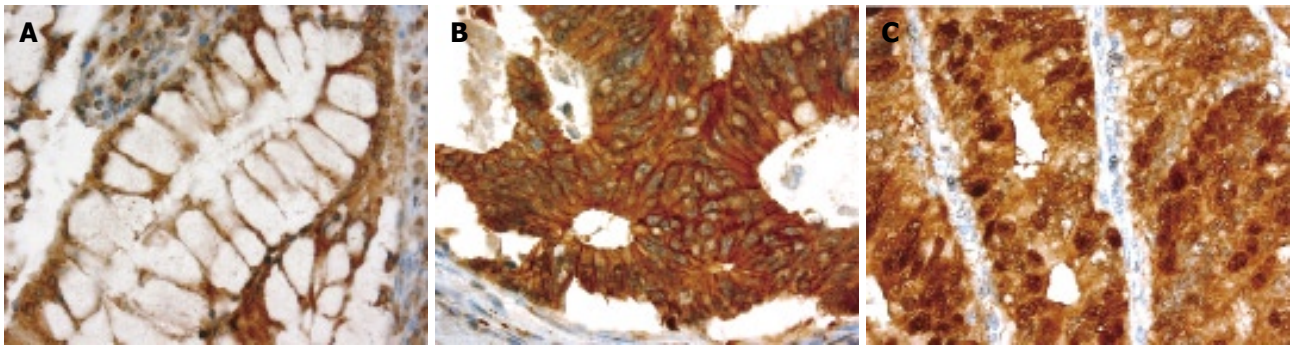

Figure 1 Different immunohistochemical (IHC) staining patterns for β-catenin in colorectal carcinomas. **A**: In normal colonic epithelium, β-catenin is predominantly expressed in the cell membrane; **B**: A medium-powered view of a colonic adenocarcinoma showing membranous and cytoplasmic expression of β-catenin; **C**: This case shows intense nuclear expression of β-catenin.

The nuclear staining index (NI) was also graded to into four categories (+++, ++, +, -): (0) Negative, only blue staining seen, (1) Weak, blue staining clearly seen through brown staining, (2) Moderate, blue scarcely seen through brown staining, nuclei appear darker than the cytoplasm, (3) Heavy staining, no blue seen through brown staining, nuclei appear darker than the cytoplasm. Three staining indexes were calculated: the membrane index (MI), cytoplasmic index (CI), and nuclear index (NI). These indices were calculated with both the intensity of staining and the fraction of positively-stained cells taken into account using the following formula:

$I = 0 * f0 + 1 * f1 + 2 * f2 + 3 * f3$

where I is the staining index and f0-f3 are the fractions of the cells showing a defined level of staining intensity (from 0 to 3). Theoretically, index scores could vary between 0 and 3[12]. The reproducibility of the evaluation of the β-catenin staining indices was tested by employing two observers (AE, AB), and the estimations showed good correlation and reproducibility (Pearson's r: MI, CI, and NI, were 0.77, 0.91, and 0.90, respectively).

Statistical analysis

Statistical analyses were performed using SPSS® (SPSS, Inc., Chicago, USA) and STATA (Stata Corp., Texas, USA) software packages (SPSS for Windows, version 14.0.1 and STATA/SE 9.2). Frequency tables were analyzed using the Chi-square test, which included the likelihood ratio (LR) or Fischer's exact test to assess the significance of the correlation between the categorical variables. Odds ratios and their 95% confidence intervals (95% CI) were calculated where appropriate, using the exact method. Differences in the means of continuous variables were analyzed using non-parametric tests (Mann-Whitney or Kruskal-Wallis) for 2- and K-independent samples, respectively. ANOVA (analysis of variance) was only used for deriving the mean values (and their SD) for each individual category. Bivariate correlation (Spearman rho) and scatterplots were used to check the correlations between two continuous variables (MI, CI *vs* DFS, DSS), controlled by linear regression analysis (R and R^2) for linearity. Univariate survival (life-table) analysis for the outcome measure (DSS, DFS) was based on Cox's method (indices treated as continuous variables), and/or using Kaplan-Meier analysis (indices with Median as cut-off). Multivariate survival analysis was carried out using Cox's proportional hazards model in a backward stepwise manner with the log-likelihood ratio (L-R) significance test, using the default values for enter and exclusion criteria. The assumption of proportional hazards was controlled by log-minus-log (LML) survival plots. In all tests, the values $P < 0.05$ were regarded statistically.

RESULTS

β-catenin expression patterns are illustrated in Figure 1. The expression pattern of β-catenin was predominantly membranous and weakly cytoplasmic in normal colonic epithelium but the pattern was cytoplasmic, membranous, or nuclear in the tumor tissue. Almost 100% of the cases showed membranous and cytoplasmic β-catenin expression, with nuclear expression being observed in 55 (58%) cases. The mean values of the three β-catenin staining indices (MI, CI, and NI) were 1.14, 1.26, and 0.80, respectively, and the median values were 1.20, 1.30, and 0.77, respectively.

We analyzed the three β-catenin staining indices in relation to all available clinical variables and tumor characteristics in univariate analyses. Using the median cut-off point, there was no correlation between β-catenin expression and most of the clinical variables (age, sex, stage, and grade). However, β-catenin expression (CI and NI) was borderline or significantly related ($P = 0.06$, $P = 0.04$, respectively) to the localization of the primary tumor, with expression being more intense in descending colon and rectum carcinomas than in lesions of the ascending and transverse colon.

There was also a marginal relation ($P = 0.086$) between NI and response to treatment in that the patients with a NI below the median had a response rate (24/48, 50%) better than those with a NI above the median (16/47, 34.0%). A direct relationship ($P = 0.068$) was found between the MI and response to treatment when the 75th percentile was used; patients with a MI > 75% had a higher response rate (9/16; 56.3%) than patients with a MI < 75% (31/79, 39.2%).

In univariate (Kaplan-Meier) survival analysis

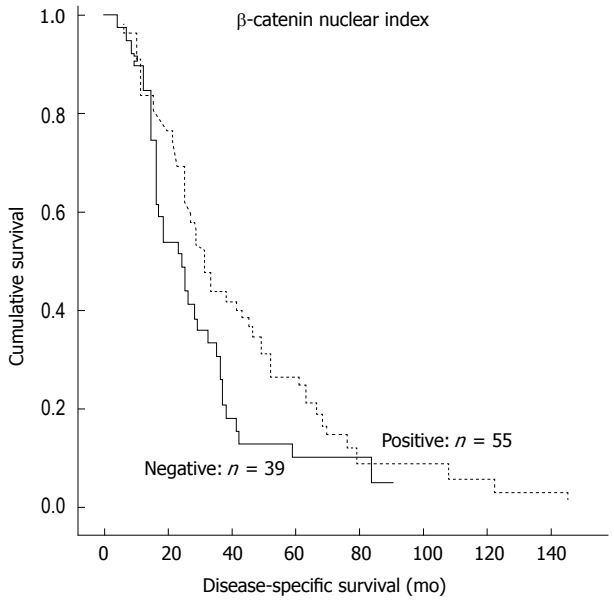

Figure 2 Disease-specific survival predicted by the nuclear index (NI) of colorectal tumors. The stippled line: nuclear expression positive. The continuous line: nuclear expression negative. The Kaplan-Meier, log rank test; $P = 0.046$. One patient died of another cause and was excluded from analyses.

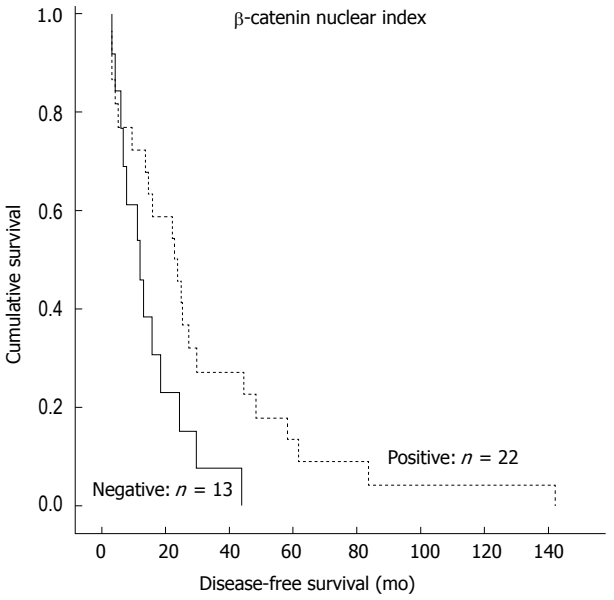

Figure 3 Disease-free survival predicted by the nuclear index (NI) of colorectal tumors. The stippled line: nuclear expression positive. The continuous line: nuclear expression negative. The Kaplan-Meier, log rank test; $P = 0.041$.

(calculable for 35 patients with stage Ⅱ or Ⅲ disease) with the median as the cut-off, only the NI was a significant predictor of more favorable disease-free survival (DFS) ($P = 0.023$). The patients with a NI above the median had longer DFS (34.2 mo) than those with a NI below the median (15.5 mo) ($P = 0.045$, ANOVA). The other indices were not significant predictors of DFS, and none of the three indices predicted disease-specific survival (DSS) in univariate analysis (Cox or Kaplan-Meier).

When the patients were stratified into two groups: nuclear expression positive ($n = 55$) and nuclear expression negative ($n = 39$), the former was a significant predictor of more favorable DSS ($P = 0.046$) (Figure 2) and DFS ($P = 0.041$) (Figure 3).

When β-catenin expression was analyzed jointly with E-cadherin expression[13] as a potential predictor of disease outcome, the combined (E-cadherin and β-catenin) cytoplasmic index did not provide any significant prognostic information.

We also reproduced the grading used by Ougolkov *et al*[7], resulting in 6/46 cases with nuclear expression at the invasive front, 20/46 cases with a diffuse nuclear pattern, and 20/46 tumors with a mixed pattern. When correlated with the treatment response and disease outcome, this grading system did not produce any results with predictive or prognostic value.

DISCUSSION

As compared with the sub-cellular distribution of β-catenin in normal colonic mucosa, neoplastic cells demonstrated a distinct shift from a membranous localization to a more widespread distribution (membranous, cytoplasmic, and nuclear) in cancer lesions. This is in line with previous reports describing β-catenin expression in cancer cells with this type of altered pattern[14,15]. For example, Wong *et al*[16] observed no nuclear β-catenin accumulation in normal tissues, whereas it was present in 8% of polyps, 92% of adenomas, and 100% of carcinomas. In the present series, nuclear expression or accumulation was observed in 48% of the cancer samples, which is in line with several other reports[7,17]. Interestingly, in 13% of the cases with nuclear expression, β-catenin was expressed by the tumour cells at the invasion fronts, a figure very similar to the 9% reported by Ougolkov *et al*[7].

We did not find significant correlations between the three β-catenin expression patterns (MI, CI, and NI) and most of the clinical variables recorded (age, sex, grade, and stage), except for tumor localization. Accordingly, β-catenin expression, both CI and NI, was more intense in carcinomas of the descending colon and rectum as compared with lesions localized in the ascending and transverse colon. Similar observations have been reported by previous studies[18-20]. There is increasing evidence to suggest that molecular mechanisms and molecular phenotypes differ in carcinomas arising in the proximal and distal segments of the large bowel[21]. The involvement of different molecular pathways in colorectal carcinogenesis is exemplified by the fact that cancers of "mutator" phenotypes preferentially occur in the proximal (right side) colon, whereas the adenoma-carcinoma sequence phenotype is characteristic of carcinomas in the distal (left side) colon and rectum[22,23]. Corresponding differences have also been shown in association with other potential prognosticators[24].

Interestingly, a marginally significant relation was observed between the NI and MI and response to treatment. Accordingly, the patients who did not respond to treatment had a NI above the median, whereas patients

who had a high MI responded better to treatment. The significance of these observations remains to be elucidated in a larger study. There is an obvious need to identify novel molecular targets for cancer therapeutics, and in this respect, the recent data showing that suppression of β-catenin can inhibit the neoplastic growth of APC-mutant colorectal cancer are of interest[25].

The correlation between β-catenin expression pattern and clinical outcome is a controversial subject. Some studies reported that cytoplasmic rather than nuclear accumulation of β-catenin is significantly related to metastasis-free survival in CRC[6]. The same was reported regarding the potential prognostic value of nuclear expression. There are studies reporting that positive nuclear expression at the invasive front of the tumor predicts shorter survival[7,19]. However, other workers failed to find any correlation between nuclear expression and survival in CRC[17,26]. In contrast, our data show that nuclear expression is a significant predictor of more favorable DSS and DFS (Figures 2 and 3). We also analyzed our samples using the same system as originally described by Ougolkov *et al*[7]. In our study, however, this special grading system did not confirm the original observation that nuclear expression at the invasive front of the tumor predicts a shorter survival[7].

There are multiple explanations for the inconsistent and, in part, discrepant results reported in different studies[6,7,17,19,26]. Such potential confounding factors might include the size of tissue samples, intrinsic tumor heterogeneity, lack of standardization in the evaluation of positive and negative results, and different immunohistochemical staining and grading methods with varying degree of sensitivity. In addition, our patients represent advanced CRC, with the majority of patients having stage Ⅳ disease, as compared with the Ougolkov study, where the majority of patients had stage Ⅱ CRC. Also, the type of treatment may have played a role in the detected relationships.

Cell-cell adhesion molecules are believed to participate in the processes of invasion, migration and metastasis[27-29]. In this regard, the E-cadherin and β-catenin complex plays a critical role in cell-cell adhesion. E-cadherin is a member of the cadherin family that mediates calcium-dependent adhesion to ensure the maintenance of a normal phenotype of epithelial cells[1,30]. β-catenin binds directly to the cytoplasmic domain of E-cadherin and to the actin microfilament network of the cellular cytoskeleton. This binding is essential for stable cell-cell adhesion[31].

It can be reasoned that altered expression of β-catenin (i.e., the shift from membranous to cytoplasmic and nuclear sites) might compromise the integrity of the E-cadherin/β-catenin complex and result in weaker cell-cell adhesion in cancer cells. Thus, it seems feasible to assess whether altered co-expression patterns of these two markers is of any predictive value in CRC. For that purpose, we combined both the membranous and cytoplasmic E-cadherin expression with membranous and cytoplasmic β-catenin expression to compare normal (MI/MI) and abnormal (CI/CI) co-expression, respectively, of these two markers as previously analysed in CRC[13]. To our disappointment, however, neither the membranous nor the cytoplasmic E-cadherin/β-catenin index (analyzed in two different modes) provided any useful information as to DFS or DSS. Thus, no added value can be obtained with analyzing E-cadherin expression together with β-catenin expression as compared to the analysis of the latter alone (Figures 2 and 3).

Taken together, the present results confirm that β-catenin expression is markedly altered in the vast majority of colorectal cancers. This shift from normal membranous expression to the cytoplasmic (CI) and nuclear (NI) patterns seems to bear some association with the localization of the primary tumour, being most pronounced in lesions of the descending colon and rectum. Although the association of CI and NI to treatment response remains unclear, NI seems to provide some prognostic value in predicting more favourable DFS and also DSS, when dichotomized as NI+/NI- expression. Many of the issues still remain unanswered, however, and additional clinical and experimental studies are needed to fully elucidate the role of β-catenin in colorectal carcinogenesis and its potential usefulness as an independent predictor of disease outcome.

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RAPID COMMUNICATION

Intrahepatic CD8$^+$ lymphocyte trapping during tolerance induction using mushroom derived formulations: A possible role for liver in tolerance induction

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Abstract

AIM: To determine the immunomodulatory effect of Shiitake (a mushroom extract), we tested its effect on liver-mediated immune regulation in a model of immune-mediated colitis.

METHODS: Four groups of mice were studied. Colitis was induced by intracolonic instillation of TNBS in groups A and B. Groups A and C were treated daily with Shiitake extract, while groups B and D received bovine serum albumin. Mice were evaluated for development of macroscopic and microscopic. The immune effects of Shiitake were determined by FACS analysis of intra-hepatic and intrasplenic lymphocytes and IFN-γ ELISPOT assay.

RESULTS: Administration of Shiitake resulted in marked alleviation of colitis, manifested by significant improvement in the macroscopic and microscopic scores, and by reduction in IFN-γ-producing colonies in group A, compared to group B mice (1.5 pfu/mL *vs* 3.7 pfu/mL, respectively). This beneficial effect was associated with a significant increase in the intra-hepatic CD8$^+$ lymphocyte trapping, demonstrated by an increased intrasplenic/intrahepatic CD4/CD8 lymphocyte ratio. These effects were accompanied by a 17% increase in the number of intrahepatic natural killer T (NKT) cells. A similar effect was observed when Shiitake was administered to animals without disease induction.

CONCLUSION: Shiitake extract affected liver-mediated immune regulation by altering the NKT lymphocyte distribution and increasing intrahepatic CD8$^+$ T lymphocyte trapping, thereby leading to alleviation of immune-mediated colitis.

Key words: Mushrooms; Colitis; Immune modulation; Shiitake; Natural killer T cell

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INTRODUCTION

Mushrooms have been valued by humans throughout history as a food product and for medical purposes[1]. Mushrooms have been used as a medicine in the Far East since ancient times. Extracts and isolated metabolites from mushrooms are known to modulate immune responses[2], resulting in enhanced innate and acquired disease resistance. The major immunomodulatory effects of the active substances derived from mushrooms include mitogenicity and activation of immune effector

cells, such as lymphocytes, macrophages, and natural killer cells. Activation of these cells can result in the production of cytokines, including interleukins (ILs), tumor necrosis factor alpha (TNF-α), and interferon gamma (INF-γ)[2]. The ability of select mushroom extracts to modulate the differentiation capacity of $CD4^+$ T cells into mature Th1 and/or Th2 subsets has been documented recently[3].

Other recent studies have suggested that these extracts may have a profound effect on Th1- or Th2-immune mediated disorders[4]. A number of bioactive molecules, mostly polysaccharides with anti-tumor properties have been identified in mushroom-derived formulations[5].

Lentinan, a (1-3)-β-glucan from *Lentinus edodes* (Shiitake), is licensed as an immunostimulatory drug[6]. Pre-treatment of mice with lentinan results in increased concentrations of TNF-α, IL-12, and IFN-γ, as well as an increase in the number of *Listeria monocytogenes-specific* CD8 T cells in the spleen. The bacterial burden in the spleen and liver of mice was reduced significantly during primary and secondary listeria infection after lentinan pre-treatment of mice. In addition, *Lentinus edodes* and its active component, the polysaccharide lentinan, have been found to be effective against several tumors, including prostate and gastric tumors, and leukemia[7,8]. However, the mechanism of action of lentinan is not clearly understood and there is currently no data on its effect on immune-mediated diseases.

The role of liver in the pathogenesis of various immune-mediated disorders is well known[9]. Liver contains a mixture of lymphocytes including both conventional T and B cells, as well as a distinct population of resident liver lymphocytes. Furthermore, liver is involved in the trapping and destruction of activated T cells[10]. As of this writing, the precise role of the liver in immune cell trapping and destruction is not fully understood, although one theory suggests that cells already in the process of apoptosis are sequestrated in the liver[11,12]. A second theory suggests that the liver plays an active role by destroying activated T cells through a local tolerance mechanism that causes clonal deletion[13,14].

The goal of the present study was to determine the effect of Shiitake on colonic inflammation in a murine model of immune-mediated colitis and to examine the role of liver in systemic tolerance induction in this setting.

MATERIALS AND METHODS

Animals

Normal inbred 2-4 mo old C57BL male mice were obtained from Harlan, Israel, and maintained in the Animal Core of the Hadassah-Hebrew University Medical School. Mice were maintained on standard laboratory feed and kept in 12-h light/dark cycles. All of the experiments were performed in accordance with the institute's ethical committee for animal handling.

Experimental design

Four groups of mice consisting of 10 animals each were studied. Mice in experimental group A were fed Shiitake extract (50 g/mouse), starting 2 d before (day 2), until 9 d after induction of colitis by intra-rectal administration of trinitrobenzene sulfonic acid (TNBS, day 9) as described previously[15]. Mice in group B were fed bovine serum albumin (BSA, 50 g/mouse). Group C was fed with Shiitake extract (50 g/mouse) from day 0 of experiment until day 9. Mice in control group D were fed with BSA, 50 g/mouse from day 0 to day 9. Mice were sacrificed on day 10.

Preparation and administration of the mushrooms

Source: Mushroom spawn (*Lentinus edodes* 4087) used in this study was purchased from Sylvan (France).

Mushroom culture: Mushrooms were grown on a 1:1 mixture of cotton and wheat straws. The straws were oven dried at 60℃ for 24 h and milled to 1-3 cm particle size. The straw mixture was wetted to 70% water content and packed into 4 kg polypropylene bags containing a microporous filter. The bags were steam sterilized at 100℃ for 2 h, then cooled to 25℃ for inoculation with 2% spawn w/w. The culture was incubated at 25℃ for 30 d. For fruiting, the temperature was reduced to 16℃ with a relative humidity of 90%, 12 h daily light and CO_2 concentration of 600-800 ppm. The fruiting bodies were then oven dried at 60℃ for 24 h and milled to 1 mm particle size.

Experimental colitis: Colitis was induced by a single enema of TNBS (Sigma, USA)[16]. Fifty milligrams of TNBS was dissolved in 20% ethanol (total volume 1 mL) and instilled through a rubber catheter 10 cm into the colon *via* the anus, without any bowel preparation. The animals were kept fasting for 24 h prior to the procedure and were anesthetized with Ketalar. After instillation, the mice were maintained in the supine position until recovery from the anesthesia to prevent immediate leakage of the instillate.

Assessment of colonic damage: Assessment of colonic damage was performed 10 d following induction of colitis[15]. Ten centimeters of the distal colonic tissue was removed and opened by a longitudinal incision and gently washed with saline. The freshly opened colonic segments were examined by an independent observer blinded to the treatment. The extent of the mucosal damage was assessed. Four macroscopic parameters were determined: degree of colonic ulceration, intestinal and peritoneal adhesions, wall thickness, and degree of mucosal edema. Each parameter was graded on a scale from 0 (completely normal) to 4 (most severe).

Colonic histology: For each animal, 6 specimens of colonic tissue from the distal 10 cm were removed for histological analysis. The tissues were fixed in formaldehyde, then sliced into 4 to 6 mm pieces, dehydrated in ethanol, embedded in paraffin wax, sectioned, and stained with hematoxylin and eosin. The degree of inflammation on microscopic sections of the colon was graded semi-quantitatively from 0 to 4.

Grade 0: Normal with no signs of inflammation; Grade 1: very low level of leukocyte infiltration; Grade 2: Low level of leukocyte infiltration; and Grade 3: High level of infiltration with high vascular density and bowel wall thickening; Grade 4: Transmural infiltration, with loss of goblet cells, high vascular density, wall thickening, and disruption of normal bowel architecture. Two experienced examiners who were blinded to the study group performed the grading.

Evaluation of the role of natural killer T (NKT) lymphocytes and the liver in CD4 trapping

Liver and spleen lymphocyte isolation: Splenocytes and liver lymphocytes were isolated as described previously with the following modifications[17]. The inferior vena cava was cut above the diaphragm and the liver was flushed with 5 mL cold PBS until it became pale in color. The connective tissue and the gallbladder were removed, and the liver was placed in a 10 mL dish containing cold sterile PBS. Splenocytes and liver lymphocytes were isolated by crushing the spleen and liver through a stainless mesh (size 60, Sigma Chemical Co., St. Louis MO)[16]. The resulting cell suspension was placed in a 50 mL tube for 3 min, washed twice with cold PBS (1250 r/min for 10 min), and all insoluble debris was removed. Cells were re-suspended in PBS, and passed through a nylon mesh pre-soaked in PBS. Unbound cells were collected and washed twice in 45 mL PBS. For liver and spleen lymphocyte isolation, the cells were suspended in a 50 mL tube containing 7 mL PBS, and underlayed with 20 mL Histopaque 1077 (Sigma Diagnostics, St. Louis, MO). The tube was centrifuged at 1640 r/min for 15 min at room temperature. Cells at the density interface were collected, diluted in a 50 mL tube, and washed twice with ice-cold PBS (pelleting at 1250 r/min for 10 min). Approximately 1×10^6 cells per mouse liver were recovered. The cell viability, assessed by Trypan blue staining was > 95%. Both splenocytes and liver-associated lymphocytes were isolated from every animal in all the experimental groups.

Flow cytometry analysis for determination of $CD4^+$, $CD8^+$ and NKT lymphocyte subsets: Following lymphocyte isolation, triplicate samples of 2×10^5 to 5×10^5 cells/500 μL PBS were placed in Falcon 2052 tubes, incubated with 4 mL 1% BSA for 10 min, and centrifuged at 1400 r/min for 5 min. The cells were resuspended in 10 μL FCS with 1:20 FITC-anti mouse CD3 antibody, 1:20 PE-anti mouse CD4 antibody, 1:20 APC-anti mouse CD8 antibody, or 1:20 FITC-anti mouse NK1.1 antibody (NKR-P1C, Pharmingen, USA), and mixed every 10 min for 30 min. The cells were washed twice in 1% BSA, and kept at 4℃ until reading. In the control group, only 5 μL of 1% BSA was added. Analytical cell sorting was performed on 1×10^4 cells from each group with a fluorescence-activated cell sorter (FACSTAR plus, Becton Dickinson). Only live cells were counted, and background fluorescence from non-antibody-treated lymphocytes was subtracted. Gates were set by forward- and side-scatter to exclude dead cells and red blood cells. Data was analyzed by the Consort 30 two-color contour plot (Becton Dickinson, Oxnard, CA) or Cellquest program.

Antigen specific IFN ELISPOT assays: IFN spot forming cells (SFC), were identified using a modified subject-specific, antigen-directed ELISPOT assay (Mabtech, Nacka, Sweden)[18]. Filtration plates (96 well), coated with high protein binding hydrophobic PVDF membrane were used (Millipore Corp., Bedford, MA, USA). The plates were coated with 1-D1K anti-IFN coating antibody (15 mg/mL, Mabtech, Nacka, Sweden) for 24 h at 4℃. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll gradient separation of 2 mL whole blood samples, collected in acid citrate dextrose tubes, and processed within 1 h. The PBMC were washed twice in RPMI 1640 with 10% fetal bovine serum. The cells were cultured in 96 well plates (1×10^5 cells/well) with RPMI 1640 and 10% FBS. Triplicate samples were prepared with 2 doses of the study drug from each subject (5 and 10 g/mL) or phytohemagglutinin (PHA, 2.5 g/mL) without antigen. Plates were incubated for 48 h at 37℃ under 5% CO_2. The plates were then washed and 100 μl biotinylated antibody (7-B6-1-biotin, Mabtech, Nacka, Sweden) at a concentration of 1 μg/mL in filtered PBS with 0.5% FBS. Plates were incubated for 3 h at room temperature. Following washing, 100 μL of streptavidin-alkaline phosphatase was added, and the plates were incubated for 90 min at room temperature. The plates were washed and substrate (BioRad, Richmond, CA) was added for 30 min, until reddish-purple spots appeared. Using a dissection microscope, dark spots, reflecting IFN-γ-secreting clones, were counted. The results were expressed as mean IFN-γ-secreting cells per 10^5 PBMC (in triplicate), after subtraction of the mean spots from wells without the study drug.

Statistical analysis

Statistical analysis was performed using the student's *t* test. $P < 0.05$ was considered significant.

RESULTS

Effect of Shiitake on survival

Shiitake administration was associated with significant improvement in the survival of animals in group A, compared with the control group B (BSA control). Of the mice in group A, 100% survived *vs* 44% in group B. All mice in control groups C and D survived.

Effect of Shiitake on severity of experimental colitis

Macroscopic scoring of colitis: The severity of colitis in mice treated with Shiitake mushroom showed marked improvement compared with mice in group B, that were fed BSA (mean macroscopic score 0.75 ± 0.2 and 3.2 ± 0.1 for groups A and B, respectively, $P < 0.005$, Figure 1A). No significant signs of colitis were observed in mice in groups C and D.

Histological assessment of colitis: Similar results

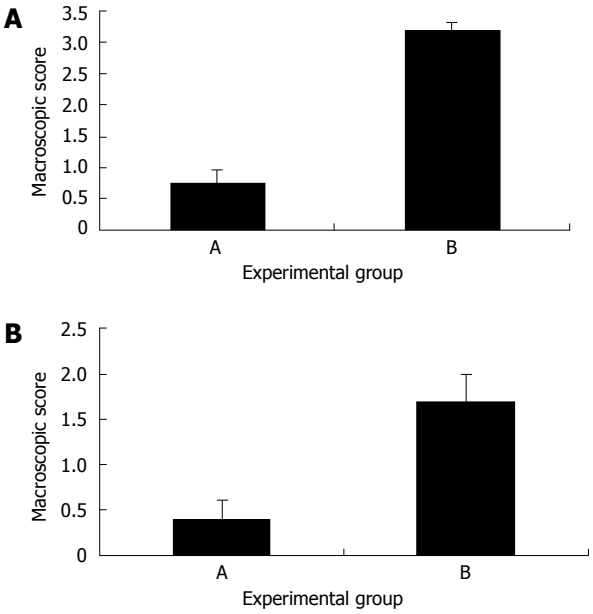

Figure 1 **A**: Effect of Shiitake administration on the macroscopic score of experimental colitis: Shiitake administration significantly alleviated the severity of colitis in group A mice compared with mice in control group B; **B**: Effect of Shiitake administration on the microscopic score of colitis: Shiitake administration alleviated the severity of colitis in group A mice compared with mice in control group B.

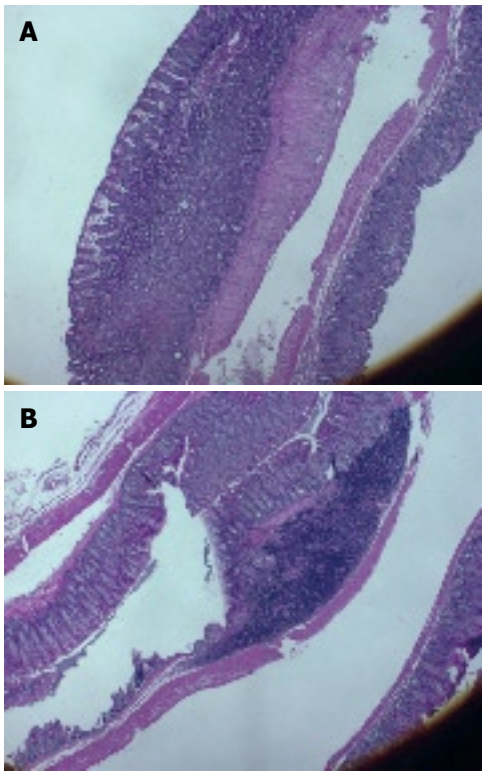

Figure 2 Histological evaluation of colonic tissues showed a marked reduction in the inflammatory response in Shiitake fed mice (**A**). By contrast, severe colitis was observed in mice in group B, manifested by inflammatory infiltration of the mucosa, and patchy necrosis of the mucosa and submucosa, with purulent and fibrinoid material extending to the muscle layer (**B**).

were obtained in the total microscopic score of colitis. The mean microscopic score was 0.4 ± 0.2 in group A *vs* 1.7 ± 0.3 in group B (Figure 1B). Histological evaluation of colonic tissues showed a marked reduction in the inflammatory response in Shiitake fed mice (Figure 2A). By contrast, mice in group B showed severe colitis, manifested by inflammatory infiltration of the mucosa, and patchy necrosis of the mucosa and submucosa with purulent and fibrinoid material extending up to the muscle layer. The muscle and serosal layers showed infiltration by acute and chronic inflammatory cells (Figure 2B).

Effect of Shiitake on NKT lymphocyte distribution

The number of intra-hepatic NKT cells was determined in all experimental groups. The number of liver NKT cells was increased in group A, compared to group B (29.1% *vs* 18.6%, Figure 3A). Liver NKT cells were also increased in group C compared with group D (32.1% *vs* 24.1%). The intrasplenic NKT cell counts, reflecting the total systemic pools of NKT cells, were also determined. A decrease in NKT cell number was observed in group A compared with group B, and in group C compared with group D (1.1 *vs* 2.4 and 1.6 *vs* 3.3, respectively). To determine whether the intra-hepatic NKT increase was part of a systemic increase in NKT cells, the intrasplenic/intrahepatic NKT cell ratio was calculated. This ratio was significantly reduced in the Shiitake fed mice (groups A and C *vs* groups B and D, Figure 3B).

Effect of Shiitake on the intrahepatic and systemic CD4/CD8 ratios

The intra-hepatic CD4/CD8 ratio was reduced significantly in mice that were fed Shiitake compared with those fed BSA (1.5 for group A *vs* 2.4 in group B, $P < 0.005$, Figure 4). A marked decrease in the intra-hepatic CD4/CD8 ratio was also noted in group C compared with the naïve group D (1.5 *vs* 2, $P < 0.005$, Figure 4). Interestingly, an opposite effect was noted when the intrasplenic CD4/CD8 ratios were calculated. Intra-hepatic CD8 trapping in Shiitake fed mice was associated with an increase in the peripheral (intrasplenic) CD4/CD8 ratio compared with BSA fed mice (4 and 2.1 for groups A and B, respectively; $P < 0.005$; Figure 4). However, even in mice without colitis, feeding Shiitake caused the peripheral CD4/CD8 ratio to decrease compared with naïve mice (2.3 in group C *vs* 3.9 in group D, $P < 0.005$, Figure 4). The intrasplenic/intrahepatic CD4/CD8 ratio was also calculated. This ratio was increased in groups A, C, D, while it was reduced significantly in group B (2.6, 1.5, 1.80 *vs* 0.9, respectively; $P < 0.005$ for all; Figure 5). The increased peripheral/liver CD4/CD8 ratio suggests CD8 lymphocyte trapping in the liver during systemic tolerance induction.

Effect of Shiitake on antigen-directed IFN ELISPOT

The number of antigen specific T-cell colonies secreting IFN-γ was reduced significantly in group A compared with group B (1.5 pfu/mL *vs* 3.7 pfu/mL, $P < 0.005$), suggesting a specific effect of Shiitake on T cells involved in Th1-mediated immune colitis.

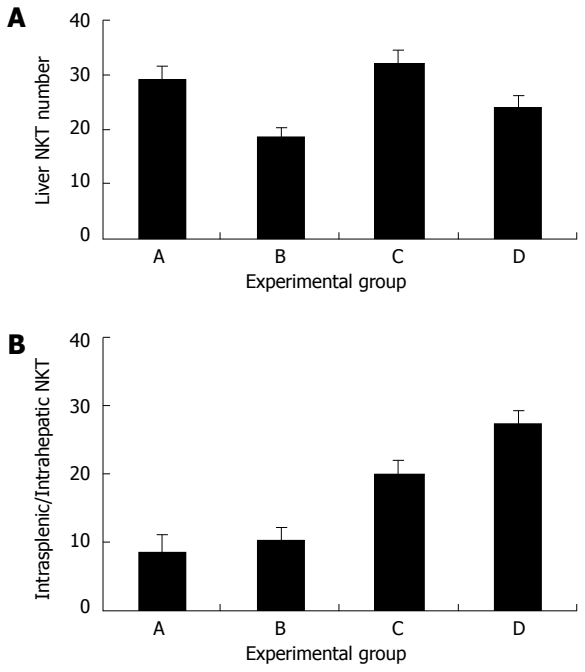

Figure 3 **A**: The effect of Shiitake administration on intrahepatic NKT cells. Liver NKT cell numbers were increased in groups A and C (which received Shiitake), compared with mice in group B and in control group D; **B**: The effect of Shiitake administration on the intrasplenic/ intrahepatic NKT cell ratio.

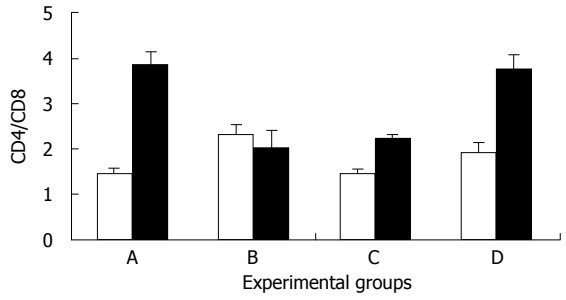

Figure 4 Effect of Shiitake administration on the intra-hepatic (open bars) and intrasplenic (black bars) CD4/CD8 ratio.

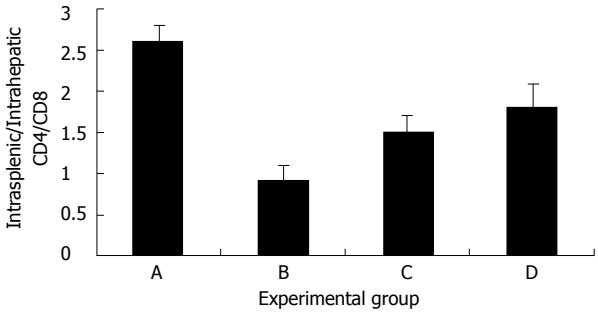

Figure 5 The effect of Shiitake administration on the intrasplenic/intrahepatic CD4/CD8 ratio: The peripheral (spleen)/intrahepatic (liver) CD4/CD8 ratio was markedly increased in groups A, C, and D. By contrast, the ratio was markedly reduced in group B.

DISCUSSION

Previous studies have shown that Lentinan, a (1-3)-β-glucan obtained from *Lentinus edodes*, has anti-tumor activity, but the purported immunomodulatory effect has not yet been tested in immune-mediated disorders[19-21]. The present study indicates that a Shiitake-derived formulation has a beneficial effect in an animal model of TNBS-induced colitis. Administration of this formulation significantly improved the survival rate, and alleviated the macroscopic and microscopic evidence (scores) of colitis. These results were associated with a dramatic reduction in the number of antigen-specific IFN-γ-producing colonies. The present study has shown that the liver has a role in mediating systemic induction of tolerance in the setting of our experimental model. This role comprises of sequestering and possibly destroying potentially harmful effector cells, resulting in disease amelioration. The systemic effect of Shiitake in reducing TNBS-induced colitis, was mediated by the trapping of CD8 T cells in the liver. Altered NKT lymphocyte distribution was also associated with the protective effect of Shiitake.

Shiitake and its active component, the polysaccharide lentinan, have previously been shown to be effective against different tumors[20,21]. Several studies have shown that these compounds are effective against gastrointestinal tumors, gynecologic tumors, as well as against leukemia and lymphoma[22,23]. In most of these studies Shiitake was used to augment the effect of other drugs[24]. It has been suggested that mushroom-derived factors do not attack the cancer cells directly, but rather produce their anti-tumor effects by activating different immune responses in the host[7,25].

Mushroom derivates can affect different parts of the immune system, including macrophage activation by induction of TNF-α, IL-6, and IL-1, dendritic cell activation, and various effects on T cells[5]. Lentinus edodes has been described as a T-cell adjuvant, skewing the Th1/Th2 balance towards Th1 through the specific induction of IL-12 from activated macrophages[26-28].

In addition to the protective effects against cancer, Shiitake has also been shown to be hepatoprotective as well as an anti-fibrotic agent. In dimethylnitrosamine-induced hepatitis, Shiitake decreased the serum aminotransferase levels by inhibiting the over-accumulation of collagen fibrils and suppressing the over-expression of genes for smooth muscle α-actin and heat shock protein-47[29]. Shiitake also inhibited, in a dose-dependent manner and without cytotoxicity, the morphologic changes and proliferation of isolated rat hepatic stellate cells (HSCs), which play a central role in liver fibrosis[29]. Interestingly, the hepatoprotective effects of Shiitake were also observed after oral administration[30]. In another model, the D-galactosamine (GalN)-induced liver injury in mice, oral administration of Shiitake decreased the release of aminotransferases and reduced the degree of histological injury[30].

Inflammatory bowel disease (IBD) is a chronic, relapsing and remitting condition of unknown etiology that exhibits a variety of autoimmune features[31,32]. Studies in animal models and in humans implicate an abnormal intestinal epithelial cell barrier function, excessive production of Th1 or Th2 cytokines, and the

unrestrained activation of $CD4^+$ $TCR\alpha\beta^+$ T cells in the pathogenesis of this disorder[32]. Induction of systemic tolerance towards disease-associated antigens has been validated as a method to alter the immune response and alleviate colitis in both animals and humans[17,18].

Rectal administration of TNBS in mice induces chronic intestinal inflammation similar to that seen in Crohn's disease in humans. Stimulated cells in the inflamed mucosa produce increased amounts of IFN-γ. IL-2, and IL-12, along with reduced IL-4 expression[33]. Administration of low dose colitis-extracted protein has been shown to inhibit the host colonic inflammatory response and to alleviate colitis in this model[17]. Tolerance induction led to an immunological shift from a pro-inflammatory Th1 type response to an anti-inflammatory Th2 type.

NKT regulatory lymphocytes differentiate through thymic and extrathymic pathways[34]. These cells are characterized as $CD4^+$ or $CD4^-CD8^-$ and $CD16^-$, express αβ TCRint, and share the surface molecules with NK cells, including NK1.1 and CD122[35]. NKT cells exist in low numbers in the peripheral blood as well as in most other tissues, but are abundant in the liver[35]. The expression of NK1.1-CD1 ligand in the liver is likely responsible for this phenomenon. Upon stimulation, these cells produce significant quantities of IL-4 and IFN-γ, and exhibit enhanced cytolytic activity[36]. Our group and others have recently shown that this subset of lymphocytes may play an important role in the induction of peripheral tolerance[33,37]. Induction of peripheral tolerance *via* oral administration of an antigen or FK506 treatment, have both been associated with a significant increase in NKT LAL production and cytotoxic activity[15]. Relevant to this study, NKT lymphocytes have also been shown to play an active role in the immune modulation of experimental colitis. Adoptive transfer of tolerized NKT cells mediate the transfer of tolerance to recipient mice and prevent the induction of disease[15].

In the present study, the beneficial effect of Shiitake was associated with increased number of liver NKT cells irrespective of the induction of colitis. This change was accompanied by a systemic decrease in NKT cell number in treated animals. To determine whether the intra-hepatic NKT increase was part of a systemic increase in NKT cells or retention of these cells in the liver, the intrahepatic/intrasplenic NKT cell ratio was calculated. While no overall increase in the number of systemic NKT cells was found, this ratio was increased significantly in Shiitake treated mice. Thus, NKT residency in the liver was increased. Taken together, these data suggest that the Shiitake-derived formulation used in the present study has a substantial effect on NKT regulatory lymphocytes. As such, such formulations may carry the potential to alleviate similar NKT-dependent disorders.

In addition to the observed changes in NKT cells, the beneficial effect of Shiitake was associated with increased intrasplenic/intrahepatic ratio of CD4/CD8, suggesting a preferential trapping of CD8 lymphocytes in the liver during systemic tolerance induction. This effect was similar to that observed in other previously described methods of tolerance induction, where the liver was shown to play an active role[9,10]. Similar to its effect on NKT regulatory cell distribution, the effect of Shiitake on intrahepatic lymphocyte sequestration was observed in treated animals whether or not colitis was induced. This finding suggests that Shiitake acts as a genuine immune modulator in both healthy and disease conditions, although a more pronounced effect was noted in disease states.

In summary, the Shiitake-derived formulation had a favorable effect on immune mediated colitis. This effect was associated with alterations in the NKT lymphocyte distribution, and on intra-hepatic CD8 lymphocyte trapping during tolerance induction. Further identification of the specific effects caused by several mushroom derived compounds may facilitate the development of oral administration of these products for the treatment of immune-mediated disorders, including IBD.

COMMENTS

Background

Natural killer T (NKT) lymphocytes play a regulatory role in various immune-mediated disorders. To determine the immunomodulatory impact of Shiitake we tested its effect on liver-mediated immune regulation in a model of immune-mediated colitis. Shiitake extract affected liver-mediated immune regulation by altering NKT lymphocyte distribution and increasing intra-hepatic $CD8^+$ T lymphocyte trapping, thereby leading to alleviation of immune-mediated colitis.

Research frontiers

The liver is a site for lymphocyte clearance, and plays an important role in determining the $CD4^+/CD8^+$ balance during tolerance induction.

Innovations and breakthroughs

Shiitake extract altered NKT lymphocyte distribution and increased intra-hepatic $CD8^+$ T lymphocyte trapping.

Applications

Shiitake extracts can serve as immune modulatory tools in various immune mediated disorders.

Peer review

The present study looked at the role of mushroom extract on tolerance induction, using an established model of TNBS colitis. It was observed that there was alleviation of microscopic and macroscopic colitis and better survival with Shiitake treatment, accompanied with changes in intra-hepatic NKT, CD4, CD8 populations with reduced IFN secreting colonies in the peripheral blood. The authors concluded that Shiitake modulates the immune response in the liver to a more tolerogenic state, and results in improvement of the colitis.

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RAPID COMMUNICATION

Measurement of circulating levels of VEGF-A, -C, and -D and their receptors, VEGFR-1 and -2 in gastric adenocarcinoma

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Abstract

AIM: To analyze the serum levels and prognostic significance of vascular endothelial growth factor (VEGF) -A, -C, and -D, and their receptors, VEGFR-1 and -2 in gastric adenocarcinomas.

METHODS: The serum levels of VEGF family members were measured in 76 control subjects and 76 patients with gastric adenocarcinoma using an enzyme-linked immunosorbent assay (ELISA). These measurements were correlated with clinco-pathological features and survival rates.

RESULTS: The serum levels of VEGF-A and its receptor, VEGFR-1, were significantly higher in patients with gastric cancer than in healthy donors (t = 2.3, P = 0.02 and t = 4.2, P < 0.0001, respectively). In contrast, the serum levels of VEGF-D were significantly higher in control subjects than in patients (t = 2.9, P = 0.004). There was no significant difference in serum levels of VEGF-C and VEGFR-2 between patients and controls. VEGF-C was associated with advanced tumor stage and presence of metastasis. VEGFR-1 was associated with metastasis, advanced overall stage, tumor differentiation and survival. VEGFR-2 levels were associated with poor tumor differentiation. There was no significant prognostic value for any of the VEGF family members or their receptors except for VEGFR-1 where high levels were associated with a poor overall survival.

CONCLUSION: Serum VEGF levels vary significantly in the same cohort of patients with variable clinico-pathological features and prognostic values. The simultaneous measurement of VEGF receptors levels in sera may overcome the limitations of a single biomarker assay.

Key words: Gastric cancer; Serum; Vascular endothelial growth factor; Oman

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INTRODUCTION

Gastric cancer is the second most common cancer worldwide and remains a global health burden[1]. The prognosis of patients with gastric cancer has been shown to be influenced by established surgical-pathological features, such as pathological stage, location of the tumor, and histological type and grade of the tumor[2-4]. In comparison, the intense search for predictive molecular biomarkers has not yet translated into clinical use[5-7].

There is growing recognition of the central role that the vascular endothelial growth factor (VEGF) family plays in angiogenesis in which the formation of new blood vessels is necessary for the growth and spread of tumors[8]. The VEGF family consists of seven members-VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E,

VEGF-F, and placental growth factor (PlGF)-which share eight cysteine residues in a VEGF homology domain[9,10]. The members act through specific tyrosine kinase receptors, VEGFR-1, -2 and -3. VEGF-A acts through VEGFR-1 and -2 receptors, VEGF-C and -D act through VEGFR-2 and -3[9,11].

Studies have shown an association between intratumoral microvessel density and tumor aggressiveness in gastric cancer[12,13]. Among angiogenic stimulators, VEGF-A plays an essential role in both vasculogenesis and angiogenesis[14]. Serum concentrations of VEGF-A have been examined in patients with gastric cancer and a relationship between the serum concentration of VEGF and metastasis and/or poor outcome has been demonstrated[15,16]. However, the significance of other VEGF family members (VEGF-B, -C, and -D) in tumor angiogenesis and metastasis are not fully demonstrated[17]. Recently, the prognostic importance of serum concentrations of other VEGF family members (VEGF-C and -D and their receptors) in gastric cancer has been reported[18,19]. Overall, these studies were limited to one or two VEGF family members and their receptors and did not examine the full profile of expression of these proteins in the same patient[14,15,18].

In the current study, we evaluated the serum levels of VEGF-A, -C, and -D, and their receptors, VEGFR-1 and -2 in both healthy controls and gastric cancer patients. We then correlated these serum levels with clinico-pathological features and patient survival rates.

MATERIALS AND METHODS

Study subjects

The study population consisted of a series of unrelated patients diagnosed with gastric cancer in three main hospitals in the Sultanate of Oman (Sultan Qaboos University Hospital, Royal Hospital, and Sohar Hospital). The control group was composed of subjects of the same ethnic and geographical origin as the patients. The Medical Research and Ethics Committee of the College of Medicine of Sultan Qaboos University approved the study design. The study subjects gave an informed consent prior to participation in the study.

Serum VEGF family level assay

Peripheral venous blood samples were obtained prior to any treatment and allowed to clot at room temperature and centrifuged at 2000 *g* for 10 min. Sera were separated, aliquoted, and stored at -70℃ until assay. Serum VEGF concentrations were determined using a commercially available enzyme-linked immunoassay (ELISA) designed to measure VEGF-A (VEGF 165), VEGF-C, VEGF-D, VEGFR-1, and VEGFR-2 levels (Quantikine, R&D Systems Europe, Abingdon, UK). Assays employed the quantitative sandwich enzyme immunoassay and were performed according to the manufacturer's procedure.

Statistical analysis

The significance of differences in the levels of various VEGF molecules between patients and controls was studied using the unpaired *t* test and further confirmed with Mann-Whitney *U* test. The significance of the correlations between the expression of various VEGF proteins and clinico-pathological features was evaluated by the Spearman's correlation test.

Overall survival rates were determined from the time of biopsy-proven diagnosis until either the time of death or last known follow-up examination. The dates of death were obtained from either medical records or phone contact. The Kaplan-Meier method was used to estimate overall survival time, and the statistical significance was determined by log-rank test. Backward conditional Cox proportional hazards regression model was utilized for multivariate analyses where age (≤ 40 years, > 40 years), gender, proximal or distal location, histological classification (intestinal *vs* non-intestinal), T stage (1 + 2 *vs* 3 + 4), presence or absence of lymph node metastasis, overall all stage (Ⅰ + Ⅱ *vs* Ⅲ + Ⅳ), tumor differentiation (well *vs* moderate + poor), all the VEFG factors were included. *P* values less than 0.05 were considered statistically significant. Analysis of data was performed using SPSS 10.0 software.

Table 1 Serum concentrations (pg/mL) of VEGF-A, -C, and -D, and VEGFR-1 and -2 in patients with gastric adenocarcinoma and control subjects (*n* = 76)

	Patients	Control	*P*
VEGF-A	585.7 ± 408.4; 469.3[1] (44.1-1927.7)	444.9 ± 328.9; 346.9 (31.1-630.6)	0.02
VEGF-C	6141.0 ± 2456.1; 5858.4[1] (970.4-12158.9)	6067.8 ± 2219.3; 5872.3 (711.7-12158.9)	0.88
VEGF-D	483.0 ± 259.3; 428.9[1] (205.7-1355.2)	671.1 ± 501.8; 605.9 (205.7-3859.7)	0.004
VEGFR-1	873.7 ± 360.8; 1026.4[1] (279.4-1767.6)	645.3 ± 297.9; 475.4 (224.1-1333.6)	0.0001
VEGFR-2	9266.5 ± 2 111.1; 9382.3[1] (4726.1-15427.3)	9420.3 ± 1840.7; 9386.2 (5727.9-15433.3)	0.45

[1]Refers to mean ± SD; median (range).

RESULTS

A total of 76 gastric cancer patients and 76 unrelated controls were included. The age range for the participants included in the study was 21-82 years; the mean and standard deviation of ages for the patients and controls were 55.7 ± 11.3 and 38.5 ± 10.2 years, respectively. The proportion of males (*n* = 41) and females (*n* = 35) were equal in both groups. Fifty four patients received either chemotherapy alone or chemoradiotherapy.

The serum values of VEGF-A, -C, -D, and VEGFR-1 and -2 in patients and controls

Table 1 shows the mean, standard deviation, median, and ranges of serum levels of VEGF-A, -C, and -D, and VEGFR-1 and -2 in patients with gastric cancer and controls. The serum VEGF-A and VEGFR-1 levels were significantly higher in patients with gastric cancer than

Table 2 Associations between serum concentrations (pg/mL) of VEGF-A, -C, and -D, and VEGFR-1 and -2 and clinco-pathological features in 76 patients of gastric adenocarcinoma and 76 control subjects (mean ± SD)

Pathological	*n*	VEGF-A	VEGF-C	VEGF-D	VEGFR-1	VEGFR-2
Site						
Distal	43	522.9 ± 371.6	6035.5 ± 2544.3	474.1 ± 276.5	863.0 ± 380.3	9058.9 ± 2140.2
Non-distal	33	667.5 ± 444.4	6278.5 ± 2368.0	494.6 ± 238.6	887.7 ± 341.7	9537.0 ± 2073.7
Classification						
Intestinal	40	586.0 ± 381.1	5895.3 ±2315.7	472.3 ± 216.9	872.8 ± 361.7	8982.6 ± 2408.5
Diffuse or mixed	36	585.5 ± 436.4	6362.2 ± 2585.0	492.6 ± 367.2	874.6 ± 366.7	9522.0 ± 1795.4
T stage+						
T1 + T2	12	635.6 ± 291.3	4843.4 ± 1682.9	465.3 ± 248.0	661.4 ± 383.3	8144.9 ± 1804.8
T3 + T4	46	484.1 ± 377.1	6378.0 ± 2345.8[a]	501.9 ± 286.4	872.3 ± 364.6	9344.9 ± 2254.5
Lymph node+						
Negative	8	597.4 ± 476.5	5450.7 ± 2381.5	397.3 ± 116.9	717.5 ± 364.9	8843.8 ± 2419.8
Positive	50	503.2 ± 346.2	6065.6 ± 2367.4	514.1 ± 291.8	828.8 ± 377.8	9065.2 ± 2076.8
Metastasis						
Absent	42	517.0 ± 331.5	5918.8 ± 2456.4	493.4 ± 283.9	779.1 ± 396.5	9007.8 ± 2297.8
Present	34	670.5 ±478.8	6415.5 ± 2464.3	470.1 ± 228.8	990.7 ± 277.1[a]	9586.1 ± 1838.1
Stage						
1 + 2	13	586.5 ± 400.1	4848.8 ± 1948.7	458.6 ± 225.1	633.1 ± 365.1	8785.6 ± 2082.9
3 + 4	63	585.5 ± 413.3	6407.7 ± 2477.9[a]	488.1 ± 267.1	923.4 ± 343.4[b]	9365.8 ± 2119.7
Differentiation						
Well	6	335.9 ± 310.5	4386.9 ± 2653.1	363.3 ± 102.0	595.5 ± 341.1	7265.1 ± 2188.0
Moderate + poor	70	607.1 ± 410.4	6291.4 ± 2399.2	493.3 ± 266.4	897.6 ± 355.8[a]	9438.1 ± 2029.6[a]

[a]$P < 0.05$ and [b]$P < 0.01$ *vs* determined in patients who had surgical resections ($n = 58$).

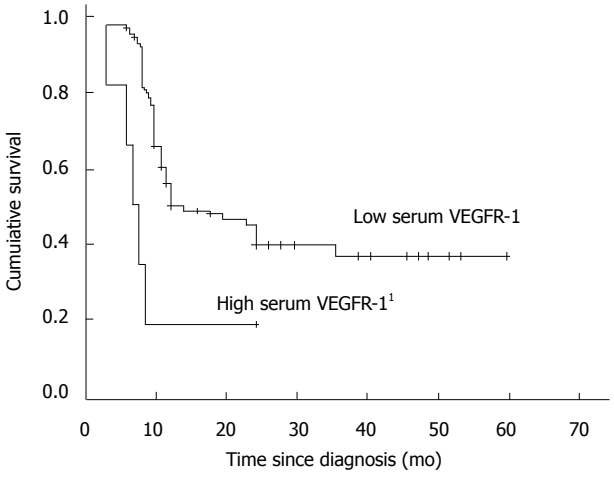

Figure 1 Kaplan-Meier survival curves in relation to serum VEGFR-1 levels in 76 patients with gastric adenocarcinoma. [1]Elevated serum VEGFR-1 levels were defined as greater than the 95th percentile in the healthy control group.

in healthy donors ($P = 0.02$ and 0.0001, respectively). Interestingly, the serum levels of VEGF-D were higher in controls than in patients with gastric cancer. There was no significant difference in serum levels of VEGF-C and VEGFR-2 between patients and controls.

The association between serum values of VEGF-A, -C, -D, and VEGFR-1 and -2 and clinico-pathological features

Table 2 shows the association between serum values of VEGF-A, -C, -D and VEGFR-1 and -2 and clinico-pathological features. VEGF-C was associated with advanced tumor stage and presence of metastasis. VEGFR-1 was associated with metastasis, advanced overall tumor stage, and tumor differentiation. VEGFR-2 levels were associated with poor tumor differentiation.

Correlation between circulating VEGF levels and patient survival

Elevated serum VEGF levels were defined as greater than the 95th percentile in the healthy control group as previously described[15]. There was no significant predictive value for any of the VEGFs or their receptors (data not shown) except for VEGFR-1 as shown in Figure 1. The median survival time for patients with high and low levels of VEGFR-1 was 5 mo (95% CI 2.6-7.4) and 13 mo (95% CI 1.4-24.6), respectively. The 5-year survival rates were 20% and 36%, respectively ($P = 0.02$). Multivariate regression analysis showed that only advanced stage Ⅲ and Ⅳ (Hazard ratio 6.7; 95% CI, 2.2 -20.0; $P = 0.001$) to be a significant independent factor.

DISCUSSION

Studies on the role of the VEGF family in gastric cancer have focused on individual VEGF members and their respective receptors[14,15,18]. The evaluation of the simultaneous expression of VEGF family members and their receptors may provide more accurate prognostic information[17]. Therefore, we have investigated the serum levels of VEGF-A, -C, -D, and VEGFR-1 and -2 and studied their predictive and prognostic significance in the same group of patients.

In the present study, the VEGF-A and VEGFR-1 levels are higher in patients with gastric cancer than in healthy controls, which is consistent with results from other studies[15,20,21]. However, we found that serum VEGF-D levels were higher in the control group than in patients with gastric cancer. George *et al*[12] studied VEGF-A, -C, and -D mRNA tissue expression in colorectal cancer progression, demonstrating that VEGF-D mRNA remained at persistently low levels in carcinomas compared with adenomas, and that

both VEGFR-2 and VEGFR-3 mRNA expression levels remained constant. This result suggested that VEGF-D acts as a competitive agonist with VEGF-A and VEGF-C; a decrease in VEGF-D levels may allow increased access of VEGF-A and VEGF-C, which are more potent angiogenic cytokines than VEGF-D, to the two VEGF receptors[22]. It has been previously shown that tissue expression of certain VEGF family members correlates with their serum levels[18]. Therefore, the advanced tumor stage presentation that predominated in the current cohort of patients may have led to the differential expression of the VEGF family members, further emphasizing the importance of the simultaneous measurement of these factors.

The correlation of markers of angiogenesis and clinico-pathological features further highlights the complex interaction between various factors and their respective receptors[16,23]. Whereas VEGF-A and -D did not correlate with any of the clinico-pathological features studied, it is interesting to note the strong association between VEGFR-1 and advanced stage cancer, the presence of metastasis, and poor tumor differentiation. VEGF-C was a serum biomarker for advanced tumor stage and poor differentiation, whereas VEGFR-2 correlated with poor tumor differentiation. It is interesting that serum VEGF-C and -D levels did not correlate with lymph node metastasis, a result that has been shown previously[18]. This finding is most likely due to the fact that most patients included had lymph node metastasis and, therefore, such differences could not be found (Table 2).

The available data on the prognostic significance of serum VEGF levels in malignancies is controversial[24-26]. The prognostic significance of VEGF (in particular, of VEGF-A), as determined by ELISA, has been commonly studied in various tumors[24-27]. It has been shown that high serum VEGF levels correlate with poor prognosis in certain hematological malignancies[28,29]. In gastric cancer, it has been shown that serum VEGF-A levels correlated with local tumor extent, disease stage, and the presence of distant metastases, and is an independent prognostic factor for patient survival[14,15,20]. Recently, Wang *et al*[9] reported that serum VEGF-C levels might be a useful biomarker to determine the presence of lymph node metastasis in patients with gastric cancer as well as correlate with VEGF-C tissue expression[18]. Juttner *et al*[8] showed that both VEGF-D and VEGFR-3 are independent prognostic biomarkers to identify patients with poor prognosis after curative resection of gastric adenocarcinomas; when combined with an analysis of VEGF-C, patients with shorter survival times could be predicted[17]. In the current study, a significant correlation between VEGFR-1 and advanced tumor stage, presence of metastasis, and poor tumor differentiation in turn reflects poor survival outcome as shown in Figure 1. To our knowledge, this is the first study to report on the prognostic significance of serum VEGFR-1 levels in patients with gastric cancer. It has been suggested that the VEGFR-1/VEGF ratio in sera may be of greater prognostic value than VEGF levels alone[28], hence, overcoming the limitations of a single biomarker assay[27,30].

In conclusion, we have demonstrated that there are significant variations in VEGF serum concentrations when measured simultaneously that correlate with important variations in clinico-pathological features and prognostic significance. Future work will address the application of this concept on a larger cohort of patients to generate a predictive profile that examines the simultaneous expression of these proteins at the tissue level.

COMMENTS

Background

The prognostic importance of serum concentrations of vascular endothelial growth factor (VEGF) family members in gastric cancer has been reported. Overall, these studies were limited to one or two VEGF family members and their receptors and did not examine the full profile of expression of these proteins in the same patient. In the current study, authors evaluated the serum levels of VEGF-A, -C, and -D, and their receptors, VEGFR-1 and -2 in both healthy controls and gastric cancer patients. They then correlated these serum levels with clinico-pathological features and patient survival rates.

Research frontiers

To date, the prognostic value of simultaneous measurement of serum concentration several VEGF family proteins in gastric cancer remains unclear. The variation of serum concentration of various VEGF family members in the same patient may provide useful predictive and prognostic tool.

Innovations and breakthroughs

In the present study, the authors measured the serum levels of VEGF-A, -C, and -D, and their receptors, VEGFR-1 and -2. This is the first study that measures serum level of VEGF family members simultaneously. The serum levels of VEGF-A and its receptor, VEGFR-1, were significantly higher in patients with gastric cancer than in healthy donors. In contrast, the serum levels of VEGF-D were significantly higher in control subjects than in patients. VEGFR-1 was associated with metastasis, advanced overall stage, tumor differentiation and survival. The result suggests that levels of VEGF family members may vary significantly and that simultaneous pre-operative measurement may provide a more accurate prognostic tool.

Applications

The results of the present study demonstrate the variation in VEGF family members when measured simultaneously. The measurement of all these proteins may provide more accurate predictive and prognostic information.

Terminology

Angiogenesis is a physiological process in the body that involves the formation of new blood vessels. VEGF family plays an important role in angiogenesis. The VEGF family consists of seven members-VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F, and placental growth factor. The members act through specific tyrosine kinase receptors-VEGFR-1, -2 and -3. VEGF-A acts through VEGFR-1 and -2 receptors; VEGF-C and -D act through VEGFR-2 and -3.

Peer review

This is an interesting paper. Authors analyzed the serum levels and prognostic significance of VEGF-A, -C, and -D, and their receptors, VEGFR-1 and -2 in gastric adenocarcinomas.

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RAPID COMMUNICATION

Increased intestinal macromolecular permeability and urine nitrite excretion associated with liver cirrhosis with ascites

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Abstract

AIM: To determine intestinal permeability, the serum tumor necrosis factor (TNF)-α level and urine nitric oxide (NO) metabolites are altered in liver cirrhosis (LC) with or without ascites.

METHODS: Fifty-three patients with LC and 26 healthy control subjects were enrolled in the study. The intestinal permeability value is expressed as the percentage of polyethylene glycol (PEG) 400 and 3350 retrieval in 8-h urine samples as determined by high performance liquid chromatography. Serum TNF-α concentrations and urine NO metabolites were determined using an enzyme-linked immunosorbent assay (ELISA) and Greiss reaction method, respectively.

RESULTS: The intestinal permeability index was significantly higher in patients with LC with ascites than in healthy control subjects or patients with LC without ascites (0.88 ± 0.12 *vs* 0.52 ± 0.05 or 0.53 ± 0.03, $P < 0.05$) and correlated with urine nitrite excretion ($r = 0.98$). Interestingly, the serum TNF-α concentration was significantly higher in LC without ascites than in control subjects or in LC with ascites (198.9 ± 55.8 pg/mL *vs* 40.9 ± 12.3 pg/mL or 32.1 ± 13.3 pg/mL, $P < 0.05$). Urine nitrite excretion was significantly higher in LC with ascites than in the control subjects or in LC without ascites (1170.9 ± 28.7 μmol/L *vs* 903.1 ± 55.1 μmol/L or 956.7 ± 47.7 μmol/L, $P < 0.05$).

CONCLUSION: Increased intestinal macromolecular permeability and NO is probably of importance in the pathophysiology and progression of LC with ascites, but the serum TNF-α concentration was not related to LC with ascites.

Key words: Intestinal permeability; Tumor necrosis factor-α; Nitric oxide; Liver cirrhosis; Ascites

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INTRODUCTION

It has been shown that the gut, as a reservoir of enteric bacteria in the body, plays a protective role as mucosal barrier function, immunoglobulin secretion, and local and systemic macrophage system, but under liver cirrhosis (LC) with portal hypertension a correlative connection between liver damage and the functional activity of the intestine with mucosal abnormalities exist[1-3]. Increased intestinal permeability (IPI) with bacterial translocation and endotoxemia have been implicated

in the pathogenesis of chronic liver injury and as contributory factors in the development of dangerous complications, such as encephalopathy and bacterial infections in LC[4]. However, other investigators have suggested that intestinal permeability is probably of limited importance in the pathophysiology of bacterial infections in patients with LC[5]. Intestinal permeability in LC has been reported as being increased or normal[6-10]. The development of systemic endotoxemia may in turn act through the release of cytokines, to further increase intestinal permeability, impair host immunity and promote bacterial translocation from the gut, thus resulting in a vicious circle[11,12]. It has been proposed that some of these cytokines play a role in several known cirrhosis-related complications, such as hyperdynamic circulation, susceptibility to infection, and hepatic encephalopathy[13]. Tumor necrosis factor (TNF)-α is a 17 kDa cytotoxic protein produced by mononuclear cells on activation by bacterial endotoxin and tissue injury[13]. However, the TNF-α level in LC has been reported with controversial findings, as it may or may not correlate with an advanced stage of disease and a worse outcome[14-18].

Nitric oxide (NO) has a role in cirrhosis. Endotoxemia, possibly from gut-derived bacterial translocation, causes induction of NO synthase leading to increase vascular NO production, which is the primary stimulus for the development of vasodilatation in cirrhosis and its accompanying clinical manifestations[19]. While NO is an unstable molecule, one means of investigating NO formation is to measure nitrite (NO_2^-), which is one of two primary stable non-volatile breakdown products of NO. A dose dependent increase in nitrite has been demonstrated to occur when macrophages are activated with lipopolysaccharide (LPS) both *in vitro* and *in vivo*[20]. Therefore, we speculated that endotoxin mediated increases in the NO metabolite nitrite in urine are related to the magnitude of intestinal macromolecular permeability and hence to LC related complications.

Limited data exists on the state of intestinal macromolecular permeability using polyethylene glycol (PEG) (400 and 3350) in cirrhotic patients with or without ascites. To clarify the role of intestinal macromolecular permeability, the serum TNF-α level and nitrite level in urine to the development of LC with ascites, we investigated whether intestinal macromolecular permeability is altered in patients LC with or without ascites, and its relationship with the serum TNF-α level and NO metabolite level in urine.

MATERIALS AND METHODS

Patients and healthy control subjects

Participating patients and healthy control subjects were comprised of 26 patients with LC with ascites, 27 patients with LC without ascites and 26 age and sex-matched healthy individuals with a normal medical history, physical examination and blood chemistry. Subjects with known infection, gastrointestinal or renal disease or diabetes mellitus were excluded from the study. Also excluded were patients that received substances known to affect intestinal permeability test results such as lactulose, non-steroidal anti-inflammatory drugs, or alcohol, in the previous 2 wk. The Institutional Review Board of the Seonam University Health Sciences Center (Namwon, Korea) approved the study. All subjects in this study gave informed consent. The diagnosis of LC was based on the typical findings of hepatic cirrhotic appearance, splenomegaly, esophageal varices, and ascites by ultrasonography and an upper gastrointestinal endoscopy, and laboratory results (prolonged prothrombin time, hypoalbuminemia with or without elevated liver enzymes). The severity of liver disease was determined according to Child-Pugh criteria.

Measurement of intestinal macromolecular permeability

Urine samples used in this study were collected during 8 h from subjects that fasted overnight (last meal before 8 PM the day before). Subjects ingested a 100 mL test solution containing 1 g of PEG 400 and 10 g of PEG 3350 in water. Each subject ingested the PEG solution 1 h before a breakfast meal. Urine samples were been kept frozen (-20℃) until processing for analysis. In this study, we attempted to detect PEG 400 as a low molecular weight (MW) marker and 3350 as a higher MW marker simultaneously in urine samples by high performance liquid chromatography (HPLC) using evaporative light scattering detection. About 2 mL of urine was filtered through a 0.45 μm syringe filter (Nylon membrane) and stored at 4℃ until analysis. All of the 1 mL-vial urine samples for analysis were directly placed into a Waters 717+ autosampler with refrigerator (10℃). The HPLC column was a 5 μm PLRP-S 100 A column (150 mm × 4.6 mm, Polymer Laboratories, Amherst, MA USA) packed with PS/DVB polymeric beads. To remove particles in the urine sample, a disposable Security Guard kit (Phenomenex, Torrance, CA USA) was used with the HPLC column. A gradient mobile phase (acetonitrile/H_2O) for an elution of 40-60 min was used to separate efficiently all hydrophilic and hydrophobic compounds. As the HPLC eluents were controlled by a gradient controller program, we tried to set the program to allow the impurities elute first while the marker compounds (PEG 400 and PEG 3350) eluted later without peak overlap. The eluted components were analyzed by an evaporative light scattering detector (PL-ELSD 2100 under conditions of evaporation -85℃, nebulizer 85℃ and gas flow 1.0; Polymer Laboratories). Calibration curves were obtained in the range of 200-1500 mg/L for PEG 400 and 10-200 mg/L for PEG 3350, respectively. The intestinal permeability was calculated by the concentration of the PEG marker compound and total urine volume. The calculated intestinal permeability index (IPI, in %), PEG retrieval ratio, is an expression of the PEG 3350 intestinal permeability, relative to PEG 400.

Table 1 Demographics and characteristics of the subjects (mean ± SE)

	Cirrhotics with ascites (n = 26)	Cirrhotics without ascites (n = 27)	Healthy controls (n = 26)
Age (yr)	54.7 ± 9.6	53.9 ± 9.7	50.3 ± 9.2
Sex (M/F)	23/3	21/6	17/9
Etiology			
Alcohol	16	15	
Viral[1]	9	12	
Alcohol/viral[2]	1	0	
Child class (A/B/C)	1/16/9	22/5/0	
Child-Pugh score	8.8 ± 0.44[b]	6.3 ± 0.34	
Serum albumin (g/dL)	2.8 ± 0.11[b]	3.5 ± 0.12	
Serum bilirubin (mg/dL)	5.1 ± 0.92[b]	2.0 ± 0.43	
Prothrombin time (s)	15.7 ± 0.50[a]	14.3 ± 0.40	
AST (IU/L)	78.3 ± 9.45	74.8 ± 11.4	
ALT (IU/L)	34.1 ± 4.8	51.5 ±8.9	
Encephalopathy	9[a]	2	
Esophageal varix	11	9	

[1]Viral etiology-cirrhosis with ascites (HBV-6, HCV-3) and cirrhosis without ascites (HBV-8, HCV-4); [2]Viral etiology-HBV-1. [a]$P < 0.05$, [b]$P < 0.01$ *vs* cirrhosis without ascites.

Measurement of serum TNF-α

With in a 12 h period after oral administration of the PEG solution, 10 milliliters of a blood sample was taken from a forearm vein of each individual. All blood samples were anticoagulated with EDTA and then plasma was separated by centrifugation at 1600 *g* for 15 min. Plasma samples were stored at -70℃ until analysis. The serum TNF-α concentration was determined by the enzyme-linked immunosorbent assay (ELISA) technique (Quantikine® human TNF-α, R & D Systems, Minneapolis, MN USA), according to the manufacturer instructions.

Measurement of urinary nitrite excretion

About 2 mL of urine was filtered through a 10000 MW filter (Millipore Microcon YM-10) and was assayed for the NO metabolite nitrite by the Greiss reaction using Parameter TM Total NO/Nitrite/Nitrate kit (R &D Systems). The total concentration of nitrite was determined by absorbance at 540 nm after urine nitrate (NO_3^-) was converted to nitrite (NO_2^-) by the NADPH-dependent nitrate reductase.

Statistical analysis

Data are reported as mean values and standard errors (mean ± SE) or percentage according to variables. Differences among the three groups were analyzed by ANOVA. When a significant effect occurred, Scheffe post hoc comparisons were used to test differences among the means. An independent samples *t*-test was used to compare test results between two groups. A nonparametric test, the Mann-Whitney test, was used to compare independently two groups that had fewer than 10 samples. We calculated Pearson's correlation coefficient for associations between two variables. SPSS statistical software (version 11.0) was used for the statistical analysis. A two-tailed significant level of 5% was chosen as a type I error.

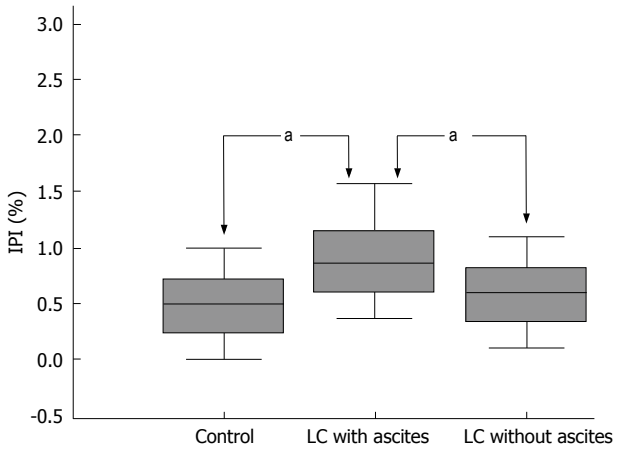

Figure 1 Polyethylene glycol retrieval ratio (PEG 3350/400) in the healthy control subjects and the cirrhotic patients. [a]$P < 0.05$.

RESULTS

Characteristics of the participating patients

There were no significant differences regarding age and gender between the cirrhotic patients with or without ascites and healthy control subjects. The distribution of causes of LC were alcohol (n = 31), viral infection (HBV 14, HCV 7; n = 21) and alcohol combined with HBV infection (n = 1). Renal function as assessed based on blood urea and creatinine levels was normal in all patients and control subjects. Details of the demographics, etiology, severity, complications of the LC and concurrent infections are outlined in Table 1.

Intestinal macromolecular permeability

Mean values for PEG 400 and 3350 retrieval were 46.5 ± 3.22 and 0.24 ± 0.03 in control subjects, 44.1 ± 5.17 and 0.21 ± 0.02 in patients with LC without ascites and 37.4 ± 3.55 and 0.31 ± 0.04 in patients with LC with ascites, respectively. The mean values for the IPI were different in patients from the healthy control subjects and patients with LC without ascites reflected the expected low diffusion of PEG 3350, being significantly higher in patients with LC with ascites (0.52 ± 0.05 and 0.53 ± 0.03 *vs* 0.88 ± 0.12, $P < 0.05$) (Figure 1). However, there was no significant difference between the healthy control subjects and patients with LC without ascites (Table 2).

A sub-analysis relating intestinal permeability to the severity of LC for all patients as indicated by the Child-Pugh class showed significant differences between class A , B and C for PEG 3350 (0.20 ± 0.02, 0.25 ± 0.03 *vs* 0.42 ± 0.08, $P < 0.05$) and IPI (0.52 ± 0.04, 0.72 ± 0.07 *vs* 1.12 ± 0.27, $P < 0.05$). According to sub-analysis relating IPI to the presence of complications of LC for patients as indicated by encephalopathy and hypoalbuminemia, there were significant differences ($P < 0.05$), but not for

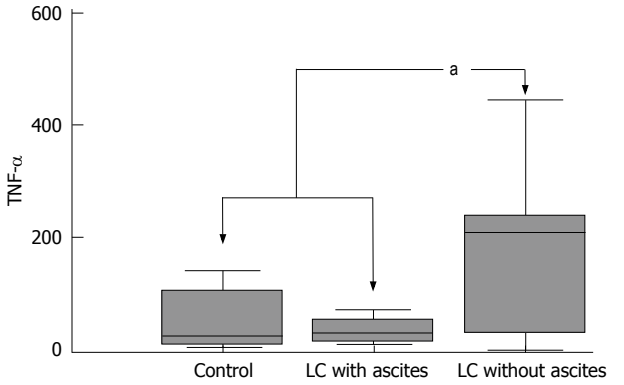

Figure 2 Tumor necrosis factor-α (TNF-α) levels in the healthy control subjects and the cirrhotic patients. [a]*P* < 0.05.

Table 2 Intestinal permeability, serum TNF-α and urine nitrite levels in the healthy control subjects and cirrhotic patients (mean ± SE)

	Cirrhotics with ascites (*n* = 26)	Cirrhotics without ascites (*n* = 27)	Healthy controls (*n* = 26)
PEG400	37.4 ± 3.55	44.1 ± 5.17	46.5 ± 3.22
PEG3350	0.31 ± 0.04	0.21 ± 0.02	0.24 ± 0.03
IPI	0.88 ± 0.12[a]	0.53 ± 0.03	0.52 ± 0.05
Nitrite	1170.9 ± 28.7[a]	956.7 ± 47.7	903.1 ± 55.1
TNF-α	32.1 ± 13.3	198.9 ± 55.8[a]	40.9 ± 12.3

PEG: Polyethylene glycol; IPI: Intestinal permeability index. [a]*P* < 0.05.

patients as indicated by a prolonged prothrombin time, esophageal varix or hyperbilirubinemia.

Serum TNF-α level

The concentration of serum TNF-α was 198.9 ± 55.8 pg/mL in patients with LC without ascites, 32.1 ± 13.3 pg/mL in LC with ascites and 40.9 ± 12.3 pg/mL in the control subjects (Figure 2). A group comparison by the Mann-Whitney test showed that the serum TNF-α level was significantly higher in patients with LC without ascites than in the control subjects and in patients with LC with ascites ($P < 0.05$) (Table 2). According to the sub-analysis relating the TNF-α level to the severity of LC for all patients as indicated by the Child-Pugh class, class A was higher than class B and C (218.8 ± 43.4 *vs* 78.9 ± 26.3 and 17.7 ± 3.1, respectively) and class B was higher than class C, and there were significant differences between them ($P < 0.05$). According to the sub-analysis relating the TNF-α level to the presence of complications of LC for patients as indicated by the presence of hypoalbuminemia, there were significant differences ($P < 0.05$), but not for patients as indicated by encephalopathy, prolonged prothrombin time, esophageal varix and hyperbilirubinemia (Table 3).

Urinary nitrite excretion

Urinary nitrite excretion was significantly increased in patients with LC with ascites as compared to patients with LC without ascites or the healthy control subjects (1170.9 ± 28.7 μmol/L *vs* 956.7 ± 47.7 μmol/L or 903.1 ± 55.1 μmol/L, P < 0.05) (Figure 3 and Table 2). According to a sub-analysis relating urinary nitrite excretion to the severity of LC as measured by the Child-Pugh class and to the presence of complications of LC for patients as indicated by the presence of encephalopathy, hypoalbuminemia, prolonged prothrombin time, esophageal varix and hyperbilirubinemia, there were no significant differences (Table 3).

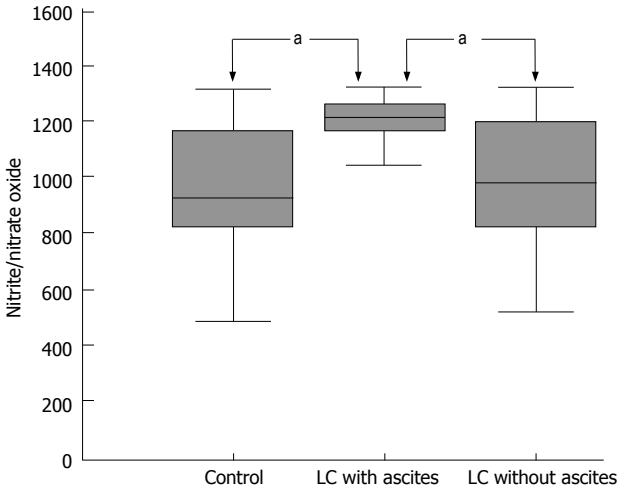

Figure 3 Urinary nitrite excretion in the healthy control subjects and the cirrhotic patients. [a]*P* < 0.05.

Correlation and statistical analysis

Patients with alcoholic *versus* non-alcoholic cirrhosis did not differ significantly ($P > 0.05$) in the intestinal macromolecular permeability (PEG 400, 3350 retrieval and IPI), serum TNF-α level and urine nitrite level. There was a positive correlation between the Child-Pugh score and increasing intestinal macromolecular permeability ($r = 0.494$ for PEG 3350 and $r = 0.447$ for IPI, $P < 0.01$), but no significant correlation between the Child-Pugh score and the TNF-α level or urinary nitrite level among patients with LC with or without ascites. No significant correlation was observed between PEG 400, 3350 percentage retrieval, IPI and the serum TNF-α level, between the TNF-α level, and urine nitrite level but there was a significant correlation between IPI and urine nitrite excretion ($r = 0.98$, $P < 0.05$) among patients with LC with or without ascites.

DISCUSSION

The concept of altered intestinal permeability is important and has been implicated in a number of pathological situations, including celiac disease associated with antigen permeability, allergic intestinal diseases such as digestive hypersensitivity, inflammatory diseases such as Crohn's disease, ulcerative colitis, acute pancreatitis, alcoholic liver disease and LC associated with substance permeability during inflammation[5,21,22].

The pathogenic mechanisms implicated in the failure of intestinal barrier in cirrhosis have not been fully elucidated as yet and remains to be investigated.

Table 3 Analysis relating intestinal permeability, nitrite level and TNF-α level to the clinical and laboratory findings in the cirrhotic patients (mean ± SE)

	n	PEG400	PEG3350	IPI (%)	Nitrite (μmol/L)	TNF-α (pg/mL)
Child-Pugh classification						
A	23	42.6 ± 5.7	0.20 ± 0.02	0.52 ± 0.04	1137.3 ± 45.4	218.8 ± 43.4[a]
B	21	38.6 ± 4.4	0.25 ± 0.03	0.72 ± 0.07	967.7 ± 67.5	78.9 ± 26.3
C	9	41.5 ± 6.0	0.42 ± 0.08[a]	1.12 ± 0.27[a]	1086.3 ± 36.4	17.7 ± 3.1
Serum albumin (g/dL)						
> 3.4	16	41.4 ± 4.9	0.23 ± 0.03	0.52 ± 0.05	1013.2 ± 65.7	183.6 ± 43.5
< 3.4	37	40.6 ± 4.0	0.27 ± 0.02[a]	0.78 ± 0.08[a]	1114.7 ± 43.6	105.8 ± 28.4[a]
Serum bilirubin (mg/dL)						
> 1.2	33	44.7 ± 4.5	0.29 ± 0.03	0.76 ± 0.09	1078.1 ± 45.7	76.6 ± 18.4
< 1.2	20	34.4 ± 3.7	0.20 ± 0.03	0.60 ± 0.07	1049.6 ± 61.2	216.3 ± 51.2
Prothrombin time (s)						
> 13	43	40.66 ± 3.6	0.27 ± 0.03	0.75 ± 0.07	1052.6 ± 40.4	121.2 ± 26.8
< 13	10	41.56 ± 7.0	0.21 ± 0.03	0.50 ± 0.07	1074.9 ± 88.8	163.8 ± 55.4
Encephalopathy						
No	42	41.58 ± 3.79	0.24 ± 0.02	0.67 ± 0.07	1097.6 ± 41.48	139.1 ± 27.52
Yes	11	37.98 ± 5.00	0.33 ± 0.04	0.82 ± 0.13[a]	1030.0 ± 77.84	91.9 ± 49.89
Esophageal varix						
No	33	42.6 ± 4.54	0.24 ± 0.03	0.72 ± 0.08	1119.9 ± 39.7	145.6 ± 33.74
Yes	20	37.86 ± 3.16	0.29 ± 0.03	0.67 ± 0.10	1007.7 ± 70.1	102.4 ± 31.30

PEG: Polyethylene glycol; IPI: Intestinal permeability index. [a]$P < 0.05$.

Toxic metabolites of alcohol are known to induce alterations of enterocyte tight junctions, which may increase paracellular permeability[23]. However, other inflammatory conditions may alter barrier integrity, as measured by increased gut permeation. An alternative mechanism may be the proinflammatory cytokines, which can be produced locally by epithelial cells or may reach the intestinal mucosa from an inflammatory focus distant from the bowel[24]. Interestingly, *in vitro* studies in cell monolayers suggest that cytokines may mediate these permeation effects by changes in the production of NO[25]. The mechanism for this effect is not known, but may involve relaxation of the cytoskeleton or oxidation/nitration of cytoskeleton proteins[26].

In the current study, PEG with different molecular masses was used to assess gut permeability, as it combines unique attributes in its chemical structure. It is non-toxic, water-soluble, as is endotoxin, and not metabolized either by the host or by intestinal bacteria[27]. After transmigration into the blood, the polar PEG-molecule is excreted with the urine. Because of its homogeneous chemical properties, its appropriately adaptable molecular mass and its linear, chain-like shape (mimicking the comparable structure of endotoxin)[28], PEG seems to be an appropriate probe for the assessment of LPS translocation through the intestine. All of these demands cannot be met by other commonly used permeability marker compounds such as mono- or disaccharides, sugar alcohols, complexes with radioactive nuclides (51Cr-EDTA, 99mTc-DTPA), proteins, or even combinations of these compounds[29].

The simultaneous use of two test marker compounds allows the expression of global intestinal permeability as an index reflecting the transfer value of the less permeable test marker (PEG 3350) relative to the most diffusible probe (PEG 400). Since pre-absorption factors such as gastric emptying, dilution by digestive secretions and post-absorption factors such as systemic distribution and renal clearance are assumed to affect both molecules equally, the value of this index should then be directly comparable from one individual to another[30].

It has been suggested that intestinal permeability markers pass through either a transcellular or a paracellular pathway. However, it is difficult to determine that endotoxins or other bacterial toxins from the gut lumen into the portal system pass paracellularly or transcellularly in cirrhotic patients, as there was no significant difference of PEG 400 and 3350 retrieval between the control subjects and cirrhotic patients with or without ascites in this study. To address this issue, further studies are needed for morphological or molecular changes of intestinal mucosa in LC during the PEG test. Distribution of PEG in ascites might have caused a lower urinary excretion rate and thus underestimated possible permeability changes in patients with ascites. However, Kalaitzakis *et al* assessing intestinal permeability with 51Cr-EDTA concluded that a loss of 51Cr-EDTA into the ascitic compartment was unlikely and paracentesis had no significant effect on the urinary 51Cr-EDTA excretion, which suggests that ascites in itself does not unduly affect the test results[5]. In the present study, ascitic fluid from three cirrhotic patients was tested for PEG and it was not detected; therefore, the possibility of lower urinary excretion rates due to distribution of PEG in ascites can be ruled out.

Previous studies have shown an association between IPI and severity of LC assessed according to the Child-Pugh classification[8,9], but other studies have failed to reproduce these results[6]. In the present study, we observed significantly higher PEG 3350 retrieval and IPI in Child-Pugh class C patients as compared with that in class A and B patients. Methodological and/or patient selection differences should be taken into account when

interpreting the results of this study.

In this study, there were no concomitant infections and a significantly higher TNF-α level in cirrhotic patients without ascites than healthy control subjects or patients with LC with ascites was seen; thus, there was a tendency for a negative correlation between the TNF-α level and Child-Pugh class in the advanced stage of LC. In advanced cirrhosis, hepatic damage and inflammation are reduced due to a decreased liver reserve and marked fibrosis, and consequently, ALT levels decrease. Additionally, diminished amounts of cytokine-producing cells such as hepatocytes and Kupffer cells may lead to a decrease of TNF-α production[15]. In LC, several inflammatory states and commonly occurring infections may be another source of TNF-α production and could explain contradicting results. Increased production of TNF-α associated with inflammation and tissue necrosis is seen not only in hepatitis, but also in other inflammatory conditions. In the present study, IPI and urinary nitrite excretion were significantly higher in patients with LC with ascites as compared to patients with LC without ascites or healthy control subjects, with a significant correlation. Since NO is thought to have a wide range of biological functions other than vasodilatation, it is likely to affect both the progress and the clinical features of LC as well as the hemodynamics in cirrhotic patients. For example, NO is a potent inducer of increased membrane permeability in the vascular endothelium and intestinal mucosa, possibly contributing to the accumulation of ascites and to bacterial translocation[31]. In the current study, although TNF-α was thought to induce NO synthesis (NOS) through the inducible NOS and endothelial NOS[32,33], there was no significant correlation between TNF-α level and NO level in LC. This is the same to some studies, where such a relation could not be observed[34,35]. It has been suggested that some other factors including TNF-α contribute to elevation of NO in LC.

A simple comparison of the published data with the findings of the present study is not easy to make. There were differences between the reported results of intestinal permeability, which means differences in the methods of assessment, including the composition of the probe solution and analytic techniques employed, as well as differences in the patient populations and in the causes and severity of LC. In conclusion, our results suggest that increased intestinal macromolecular permeability and NO are probably of importance in the pathophysiology and progression of LC with ascites, and furthermore, IPI may be a contributory factor in the development of encephalopathy in LC.

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COMMENTS

Background

Increased intestinal permeability (IPI) with bacterial translocation and endotoxemia have been implicated in the pathogenesis of chronic liver injury and as contributory factors in the development of dangerous complications, such as encephalopathy and bacterial infections in liver cirrhosis (LC). However, limited data exists on the state of intestinal macromolecular permeability using PEG (400 and 3350) in cirrhotic patients with or without ascites. To clarify the role of intestinal macromolecular permeability, the serum tumor necrosis factor (TNF)-α level and nitrite level in urine to the development of LC with ascites, the authors investigated whether intestinal macromolecular permeability is altered in patients LC with or without ascites, and its relationship with the serum TNF-α level and NO metabolite level in urine.

Innovations and breakthroughs

The authors investigated the relation between intestinal permeability in compensated and decompensated cirrhosis in relation to TNF-α and urine nitrite oxide levels. Their results suggest that increased intestinal macromolecular permeability and NO are probably of importance in the pathophysiology and progression of LC with ascites, and furthermore, IPI may be a contributory factor in the development of encephalopathy in LC.

Applications

All the findings of the current study will provide useful information for the understanding and the treatment of LC.

Peer review

This is an interesting study on a relevant topic. The main results of the study are that the increased permeability and nitrite oxide is of importance in the pathophysiology of decompensated cirrhosis.

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RAPID COMMUNICATION

Change of choline compounds in sodium selenite-induced apoptosis of rats used as quantitative analysis by *in vitro* 9.4T MR spectroscopy

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Abstract

AIM: To study liver cell apoptosis caused by the toxicity of selenium and observe the alteration of choline compounds using *in vitro* 9.4T high resolution magnetic resonance spectroscopy.

METHODS: Twenty male Wistar rats were randomly divided into two groups. The rats in the treatment group were intraperitoneally injected with sodium selenite and the control group with distilled water. All rats were sacrificed and the livers were dissected. ^{1}H-MRS data were collected using *in vitro* 9.4T high resolution magnetic resonance spectrometer. Spectra were processed using XWINNMR and MestRe-c 4.3. HE and TUNEL staining was employed to detect and confirm the change of liver cells.

RESULTS: Good ^{1}H-MR spectra of perchloric acid extract from liver tissue of rats were obtained. The conventional metabolites were detected and assigned. Concentrations of different ingredient choline compounds in treatment group *vs* control group were as follows: total choline compounds, 5.08 ± 0.97 mmol/L *vs* 3.81 ± 1.16 mmol/L (P = 0.05); and free choline, 1.07 ± 0.23 mmol/L *vs* 0.65 ± 0.20 mmol/L (P = 0.00). However, there was no statistical significance between the two groups. The hepatic sinus and cellular structure of hepatic cells in treatment group were abnormal. Apoptosis of hepatic cells was confirmed by TUNEL assay.

CONCLUSION: High dose selenium compounds can cause the rat liver lesion and induce cell apoptosis *in vivo*. High resolution ^{1}H-MRS *in vitro* can detect diversified metabolism. The changing trend for different ingredient of choline compounds is not completely the same at early period of apoptosis.

Key words: Apoptosis of liver cell; Choline compounds; Sodium selenite; *In vitro* ^{1}H-MRS; Quantitative analysis

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INTRODUCTION

Apoptosis is a programmed, active, highly selective mechanism of cell death. Multicellular organisms' apoptosis is an essential component of cellular regulation. Abnormal regulation of apoptosis can lead to disorders such as cancer[1,2].

The field of cell death research has undergone an explosion of new knowledge over the past decade. The methods to evaluate death of cells, especially in intact tissues, have led to the development of several techniques. However, the properties revealed in these assays are not always applicable to study of diversified metabolite of apoptosis at one time[3].

Nuclear magnetic resonance spectroscopy is a non-destructive and non-invasive technique that can provide complete structural analysis of a wide range of organic molecules in complex mixtures. It generates

quantitative information, as the peak intensities can be proportional to analyze concentrations[4]. Because of their low sensitivity and small magnet gaps, the early spectrometers had limited applications, primarily to synthetic chemistry[5]. Over the past three decades, however, sensitivity has increased by orders of magnitude so that this technique can be used to detect the metabolite alteration of apoptosis[6-8].

Choline compounds are one kind of biologically interesting metabolites that can be detected by ^{1}H-MRS. Declining of choline compounds is considered as a ^{1}H-NMR metabolite marker of advanced stage of apoptosis[9]. Lehtimaki *et al*[10] consider that choline compounds stay unchanged despite reduced cell density. However, how the intensity choline compounds change when apoptosis occurs is still confused.

Our hypothesis was that there is no reason for choline compounds to stay unchanged when apoptosis of liver cell occurs. The alteration of choline compounds could be observed through detailed quantitative analysis by high-resolution ^{1}H-MR spectroscopy. Therefore, the purpose of this study was to observe the liver cell apoptosis caused by the toxicity of selenium and the alteration of choline compounds using *in vitro* 9.4T high resolution magnetic resonance spectroscopy.

MATERIALS AND METHODS

Animal

Twenty male Wistar rats, weighing 280-320 g, were randomly divided into two groups (n = 10). The rats in the treatment group were intraperitoneally injected with sodium selenite liquor (Na_2SeO_3) at a dose of 20 μmol/kg and the control group with distilled water at a dose of 1 ml/kg. The rats of both groups were fasted for 12 h but with free access to water. All the rats were sacrificed after 24 h. The livers of all rats were immediately dissected. Parts of livers were frozen in liquid nitrogen and then stored at -70℃ until measured. The rest parts of livers were fixed in formalin. All animal experiments were performed according to the guidelines approved by the Ethical Committee of the Medical College of Shantou University.

In vitro ^{1}H-MR spectroscopy

Frozen liver tissue was pulverized with a pestle and mixed with a volume of 2 mmol/mL ice-cold perchloric acid. The mixture was transferred to a homogenizer and homogenized for 20 min at 4℃. The mixture was neutralized with ice-cold 3mmol/mL and 2 mmol/mL KOH and then centrifuged at 10 000 g for 30 min in order to eliminate perchlorate salts. The resulting supernatant was lyophilized and the precipitate discarded. The powder of extracts was transferred into a 5-mm NMR tube and redissolved in 500 μL D_2O containing 1 mmol/L 2,2'-3,3'-tetradeutero-trimethyl-sylilpropionate (TMSP). D_2O was added for locking signal. TMSP was used as an internal chemical shift reference at 0.00 ppm. Each sample was collected using *in vitro* 9.4T high resolution magnetic resonance spectrometer (Bruker Avance 400 MHz). Spectra of extracts were acquired with a pulse sequence with water suppression from the Bruker zgpr pulse program. Data were obtained over a 5000 Hz sweep width and digitized with 4096 data points. The number of scans was 128. Total acquisition time was 6 min. Spectra were primarily processed in the frequency domain using XWINNMR (Bruker GmBH), including fourier-transformation, phased correction and baseline correction. The chemical shift was assigned according to the internal standard TMSP and advanced analysis was then performed using MestRe-c 4.3.

Statistical analysis

The peak areas which were assigned as metabolites containing choline and TMSP and integral values were calculated separately by MestRe-c 4.3. The concentration of choline was calculated following the amended formula[11].

$$(\text{metabolite}) = \frac{\text{square(metabolite)}}{\text{square(TMSP)}} \times \frac{\text{number of protons of metabolite}}{9} \times (\text{TMSP})$$

Square (metabolite) and square (TMSP) stand for the area of peaks of choline compounds and TMSP; 9 correspond to the number of protons giving rise to the TMSP peak; (TMSP) and (metabolite) represent the concentration of TMSP and metabolite.

The concentration data were put into computer and analyzed using SPSS 13.0 software. A two-sample t test was used for comparison of choline concentration of the samples in both groups. Significance level was set at $P < 0.05$.

Histopathology

Parts of livers presenting no necrosis by visual inspection underwent histopathological examination during follow-up. Formalin fixed samples were embedded in paraffin and 4-mm sections were cut. Samples were stained with hematoxylin and eosin and examined under light microscopy.

The terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was performed using the *in situ* cell death detection kit according to Schrum LW[12]. Briefly, DNA ends were tagged with fluorescein-labeled dUTP using terminal deoxynucleotidyl transferase by incubating the samples at 37℃ in a humidified chamber. Liver sections were then incubated with anti-fluorescein-alkaline phosphatase conjugate for 30 min in a humidified chamber. Slides were incubated with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium for 20 min at room temperature and were counterstained with hematoxylin. The sections were observed under light microscopy.

RESULTS

^{1}H-MRS spectra of perchloric acid extract from rat liver tissues are shown in Figure 1. The conventional metabolites were detected and assigned. The assignments of spectra were performed following the

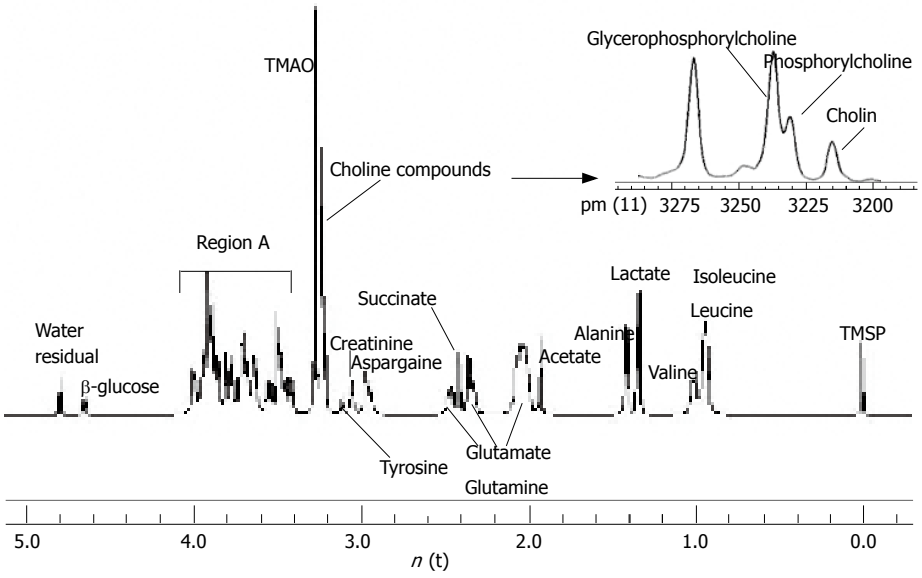

Figure 1 High-resolution proton spectra of liver tissue of a rat in control group and internal standard (TMSP). The field 0.0-5.0 ppm is shown.

Table 1 Assignments of liver metabolites from male Wistar rats

Metabolite	Chemical shift (ppm)
TMSP	0
Isoleucine and Leucine	0.87
Valine	0.96
Lactate	1.32
Lysine	1.47
Alanine	1.48
Acetate	1.92
Glutamate	2.07-2.34
Succinate	2.41
Glutamine	2.13-2.45
Aspargaine	2.95
Creatinine	3.05
Tyrosine	3.11
Choline compounds	3.20-3.23
TMAO	3.27
Region A	3.41-4.0
β-glucose	4.67
Water (residual)	4.75

TMSP: 2,2′-3,3′-tetradeutero-trimethyl-sylilpropionate; TMAO: Trimethylamine-N-oxide methyl; Region A: Glucose and amino acid CH resonances.

previous studies[13-16] and presented in Table 1. The resonances of partially megascopic region 3.20-3.27 ppm were well resolved, where $N(CH_3)_3$ signals from the compounds choline, phosphorylcholine and glycerophosphorylcholine can be separated. Following the formula, the total choline compounds and free choline concentrations were calculated. The mean concentration of total choline compounds was 5.08 ± 0.97 mmol/L in control group and 3.81 ± 1.16 in treatment group and the mean concentration of free choline was 1.07 ± 0.23 mmol/L in control group and 0.65 ± 0.20 in treatment group. The differences of the two groups were statistically significant (P = 0.05 and P = 0.00, respectively). However, there were no statistical significances if we compared the concentrations of synthetical choline, including phosphorylcholine and glycerophosphorylcholin (3.71 ± 0.74 mmol/L *vs* 3.01 ± 0.94 mmol/L, P = 0.46) between the two groups.

Although the structure of liver lobules was normal in both groups in the view of 100 times magnification, the hepatic sinus was wider in treatment group than in the control group. Cell shape was then inspected in 400 times magnification under light microscopy. The normal hepatic cells were like short shuttle while hepatic cells in treatment group were irregular and verge was not clear. Some of the cells were slightly bigger than the normal ones, but smaller ones were more often observed. We found that the cytoplasm was dyed redder than normal ones. Simultaneously, the condensed or diffuse chromatin remained randomly distributed, and the nuclear pores disappeared (Figure 2). No obviously inflamed cells could be detected under light microscope. Apoptosis of hepatic cells in both groups was confirmed by TUNEL assay. The brown ones in TUNEL assay were the apoptosis positive (Figure 3).

Although positive cells were detected in both groups, the number of positive cells was conspicuously more in treatment group than in control group. The positive cells were spread around in the control group but relatively assembled in the treatment group. Some of the apoptosis positive hepatocytes were vacuolated in treatment group. We found a very interesting phenomenon in our results: Few of apoptosis positive cells were detected in a rat of the treatment group. The ^{1}H-MRS data showed that choline concentrations were also much higher than the mean concentration of the treatment group, the concentration of total choline, free choline and synthetical choline of this rat was 5.67, 0.87 and 4.04 mmol/L, respectively.

DISCUSSION

Selenium is an essential trace element for human health. The cellular effects of selenite appear quite complex and are concentration-dependent. It is demonstrated that the serum level of selenite affects cell proliferation[17]. At intermediate concentrations, selenite appears to exert its chemopreventive activity. At higher concentrations,

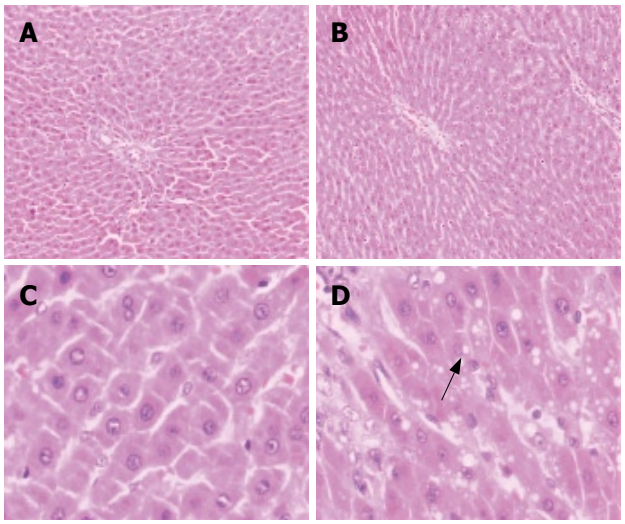

Figure 2 Light microscopy of HE stained livers. **A**: Specimen from control group (x 100); **B**: Specimen from treatment group (x 100); **C:** Specimen from control group (x 400); **D**: Specimen from treatment group (x 400). Arrowhead indicates vacuolated hepatocytes.

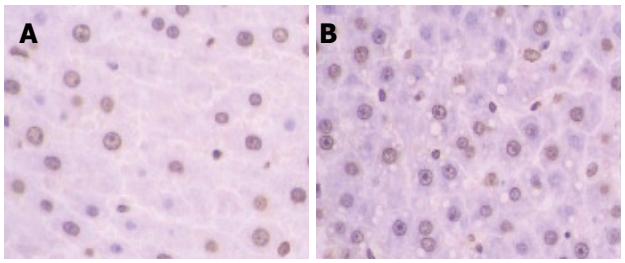

Figure 3 The brown hepatocytes showing the cell apoptosis (TUNEL staining). **A**: Specimen from control group (x 400); **B:** Specimen from treatment group (x 400).

selenite induces oxidative stress and may become toxic[18]. Many researchers focused on the induction of apoptosis by toxic concentrations of selenite. Selenocompounds have been reported to induce apoptosis in non-malignant cell lines, such as the Chang liver cells[19]. The results of our experiments also intensively suggested that high-concentration selenium was able to cause lesions in rat livers and induce apoptosis *in vivo*. However, we also observed that one rat liver in treatment group did not present obvious cell apoptosis. We think that this phenomenon might be related to the individual diversity. Some of the rats are not sensitive towards this remedy. Bollard *et al* pointed out that various intrinsic physiological factors were known to affect the metabolic composition of biological samples from healthy experimental animals. These included well-being, genetic drift, strain, hormonal differences, rate of metabolism, age, and gender[20]. It is still unclear how selenocompounds might induce apoptosis. Many potential mechanisms have been proposed, including protection against oxidative damage, modulation of metabolism of carcinogens, cytotoxicity of selenium metabolites, induction of apoptosis secondary to production of ROS, regulation of the thioredoxin redox system, regulation of the cell cycle, and inhibition of angiogenesis[21].

It has been reported that, with Se supplementation, the liver Se concentration increases disproportionately[22]. How to predict the liver lesion and inspect the efficiency and toxicity of selenocompounds is an important issue. ^{1}H-MRS is one of suitable, low-cost, and accurate methods for this study.

MR spectroscopy possesses the sensitivity required to measure subtle biochemical changes[23]. Although MR spectroscopy detects only a fairly small number of metabolites, it can still be used to monitor the activity of many cellular activities, because so many metabolic pathways are connected[24]. Choline compounds are one group of metabolites that can be detected by ^{1}H-MRS.

Choline is a nutrient essential for normal function of all cells[25]. It is a precursor not only for acetylcholine but also for phospholipids that are found in intracellular membranes and in the cell membrane[26]. The total choline peaks consist of glycerophosphorylcholin, phosphorylcholineand free choline, but the low resolution of *in vivo* spectroscopy can not identify the individual peaks from these compounds[27]. Because of the high resolution of the spectrometer which we used in this experiment, free choline is detached among the choline compounds so that we can further find out how free choline changes are when apoptosis of cells takes place separately. In our research, we found that the total choline declined when apoptosis occurred in the liver cells. This result is quite conformable with the result of Blankenberg FG and the conventional idea, but different from the results of Lehtimaki[10]. In our opinion, there are three reasons for this phenomenon: firstly, it is related to the inspected organ, which is liver but not brain. As it is known, all ingested choline and free choline generated by phospholipid metabolism enter the hepatic circulation, making the liver, where there are very active biochemical pathways for choline metabolism, a significant "sink" for choline[28]. But when a lesion arises in the liver, the liver loses the function of absorbing choline and causes choline declining. Secondly, the method used to induce liver cell apoptosis could cause this difference. One of the reasons why the selenocompounds cause cell apoptosis is that it is capable of inducing rapid superoxide generation and *p53* phosphorylation[29]. This activation can initiate mitochondrial dysfunction and result in energy insufficiency. Ultimately, it may affect the exchange of the cell containing substance, including choline compounds. This is testified by Luck *et al*[30]. Finally, cell apoptosis period is also one of the influencing factors towards the result. In our study, the apoptosis was in its early phase. At the beginning of cell apoptosis, the total choline compounds declined because free choline decreased. This was supported by our spectroscopic and light microscopic data. It is also very interesting that the total concentration of synthetical choline, including phosphorylcholine and glycerophosphorylcholin did not have statistical difference between the two groups. Energy insufficiency and activity decline may originally cause the concentration decrease of synthetical choline when apoptosis takes place in liver cells. Because of the synthetical choline supplement by membrane dilapidation

and release when the samples were mashed, the total concentration of synthetical choline finally remained fairly constant. This process of membrane perturbations is mainly the function of phospholipase A_2 activity *in vivo*[31].

In summary, high dose selenium compounds can cause lesion of rat liver and induce cell apoptosis *in vivo*. *In vitro* high resolution ^{1}H-MRS can detect diversified metabolism that can resolve in water. The data of the spectroscopy include quantitative and qualitative information. Moreover, it can repetitively and accurately evaluate the lesion of the organ at early stage. Thus, this method has a potential role in oncology, including detection of malignancy, grading of tumor, predicting and monitoring the treatment response, and identifying persistent or recurrent diseases[32,33]. In our study, we found that the changing trend for different ingredient of choline compounds is not completely the same at early period of apoptosis. Further studies are needed to know how choline compounds change at the advanced and final stage of apoptosis.

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COMMENTS

Background

The field of cell death research has undergone an explosion of new knowledge over the past decade. The methods to evaluate death of cells have led to the development of several techniques. However, the properties revealed in these assays are not always applicable to study of diversified metabolite of apoptosis at one time. Magnetic resonance spectroscopy is a non-destructive and non-invasive technique that can provide complete structural analysis of a wide range of organic molecules in complex mixtures. This technique used to detect the metabolite alteration of apoptosis has been reported. Choline compounds are one kind of biologically interesting metabolites that can be detected by ^{1}H-MRS. However, how the intensity of choline compounds changes when apoptosis occurs is still confused.

Research frontiers

The alteration of different ingredient of choline compounds could be quantitatively analyzed by *in vitro* 9.4T high-resolution ^{1}H-MR spectroscopy when apoptosis of liver cell takes place because of the toxicity of selenium. The results of present article will be helpful for further studies concerning metabolism of apoptosis.

Innovations and breakthroughs

When apoptosis of liver cell takes place, the concentrations of total choline and free choline decline, whereas the total concentration of synthetical choline, including phosphorylcholine and glycerophosphorylcholine, stays unchanged.

Applications

This study is useful to explain how apoptosis of liver cell occurs. It may also play an important role in guiding the clinical diagnosis and treatment of tumors.

Peer review

This is an interesting study, where the advanced technique of ^{1}H-NMR to detect the effect of choline compounds is paralleled to TUNEL staining of liver tissues of rats injected with selenium.

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RAPID COMMUNICATION

1,25-dihydroxyvitamin D_3 regulates LPS-induced cytokine production and reduces mortality in rats

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Abstract

AIM: To study the immunoregulatory effect of 1,25-dihydroxyvitamin-D_3 Von dominant Th1 response in rats.

METHODS: Sixty adult Lewis rats were randomized into three groups. Rats in group 1 (n=25) were treated with 1,25-$(OH)_2D_3$ first and then challenged with LPS, rats in group 2 (n=25) were treated with vehicle first and then challenged with LPS. Ten animals in groups 1 and 2 were preserved for mortality observation. The remaining animals were injected (i.p) with endotoxin, 24 h after the last administration of 1,25-$(OH)_2D_3$ and vehicle. Rats in group 3 (n=10) were treated with 1,25-$(OH)_2D_3$ only. Serum IL-12, IFN-γ, IL-2 and IL-4 levels were measured and target gene of 1,25-$(OH)_2D_3$ on Th cells was studied after 6 h. Gene abundance was verified by real-time quantitative PCR.

RESULTS: No death occurred in rats pretreated with 1,25-$(OH)_2D_3$ after LPS injection. Death occurred 9 h after LPS injection in rats pretreated with the vehicle, and the number of deaths was 5 within 24 h, with a mortality rate of 50%. There was no change in the number of deaths within 96 h. Six hours after endotoxin stimulation, serum IL-12 and IFN-γ levels decreased significantly in rats pretreated with 1,25-$(OH)_2D_3$ as compared with those in rats pretreated with the vehicle. The serum content of these two cytokines was very low in rats not challenged by endotoxin, and there was a significant difference as compared with the previous two groups.

CONCLUSION: 1,25-$(OH)_2D_3$ attenuates injury induced by the lethal dose of LPS, regulates Th1 and Th2 cells at the transcription level, and dominantly responds to cytokine production in rats.

Key words: Endotoxin; Cytokine; 1,25-dihydroxy-vitamin-D_3; Immunoregulation; Mortality

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INTRODUCTION

1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] is an active form of vitamin D, which not only regulates the dynamic balance of calcium and phosphorus metabolism but also participates in differentiation and regulation of the immune system[1,2]. *In vitro* study[3] showed that both antigen-presenting cells (APCs) and activated lymphocytes express vitamin D receptor (VDR), and that 1,25-$(OH)_2D_3$ acts on APCs (mainly dendritic cells) and helper T cells (Th) through VDR mediation[4], inhibits proliferation and differentiation of Th1 and cytokine production, and induces differentiation of Th2. The status of Th1/Th2 differentiation determines the type of immune response and the final outcome of body response[5]. Cytokine environment is the key factor for initiating Th1/Th2 differentiation[6,7].

Th1 immune response is not only associated with a variety of acute inflammatory responses but also plays a leading role in the development and progression of many autoimmune diseases and transplantation rejection[1,8-12]. Few *in vivo* studies reporting the influence of 1,25-$(OH)_2D_3$ on Th1 immune response are available, and the experimental results about cytokine regulation are conflicting or completely different[5,13-17]. The target gene in Th cells

remains almost unknown[10,17]. *E.coli* endotoxin is a potent bacterial mitogen, able to promote maturity of immature dendritic cells (DC), directly activates T cells and induces Th1 immune response[18]. We established a Th1 dominant response animal model and pre-treated it with 1,25-$(OH)_2D_3$. The results of our study showed that 1,25-$(OH)_2D_3$ was able to regulate the production of IL-12, IFN-γ and IL-4 in dendritic, Th1 and Th2 cells. The effector target point of regulation was at the gene transcription level. It is the regulation of 1,25-$(OH)_2D_3$ on T cell polarization that attenuates injury induced by the lethal dose of LPS in rats and significantly reduces the mortality of rats.

MATERIALS AND METHODS

Animals

Inbreed line Lewis rats (at the age of 3.5-4.5 mo, weighing 242 ± 14 g) were provided by Experimental Animal Center of the Chinese Academy of Medical Sciences (Beijing, China) and fed with normal chow containing 1.6% calcium, 0.9% phosphorus and 0.3% vitamin D (Nanjing Animal Technology Co., Ltd, Nanjing, China) with free access to water. The experiment protocol followed the institutional regulations of the Ministry of Health of the People's Republic of China concerning animal experimentation.

Experiment protocol

Sixty rats were randomized into three groups. Rats in group 1 (n = 25) as the study group, were administered 1,25-$(OH)_2D_3$ by gavage (GmbHcd&Go, Swiss) at 1 μg/animal for 14 d[19], rats in group 2 (n = 25) as the positive control group were administered the same dose of the vehicle for 14 d by gavage. Animals in groups 1 and 2 were injected intraperitoneally with *E.coli* 0111, B4 (Sigma, USA). Rats in group 3 (n =10) as the negative control group were administered 1,25-$(OH)_2D_3$ only by gavage at the dose of 1 μg/animal for 14 d, and injected (i.p) with the same volume of normal saline (Sigma Chemical CO., St Louis, MO, USA).

Ten animals in groups 1 and 2 were preserved for mortality observation. The remaining animals were injected (i.p) with endotoxin (10 mg/kg), 24 h after the last administration of 1,25-$(OH)_2D_3$ and vehicle. Six hours after the injection, they were anesthetized with 50 mg/kg (i.p) pentobarbital (Sigma-Aldrich, USA) and used for drawing 5mL blood from the abdominal major artery. The blood was centrifuged at 4℃ for 15 min, and the serum was stored at -80℃ for test. The spleen was removed aseptically, washed with PBS and stored in liquid nitrogen.

Enzyme-linked immunosorbent assay (ELISA)

Serum IL-12, IL-2, IFN-γ and IL-4 levels were measured with commercially available ELISA kits (Biosource CO., Camarillo, CA, USA) according to the manufacturer's instructions, and the quality control serum values were calculated.

Ca^{2+}/NF-AT signaling pathway gene array

Three spleen tissue samples were chosen randomly from rats in groups 1 and 2 for RNA extraction. UV absorption precipitation method and denaturing gel electrophoresis were used to test the quantity, quality and completion of RNA. The probe was synthesized by RT-PCR. Five μg RNA was used to prepare annealing solution and mixed with RT solution to undergo reverse transcription reaction under the action of reverse transcriptase (M1701, Promega, USA).

Chip hybridization was conducted by using Ca^{2+}/NF-AT signaling pathway gene array chip (Super Array Bioscience CO., Cat.NO.HS-022 USA) and chemiluminescent assay kit (Super Array Bioscience CO., NO.D-01) according to the manufacturer's instructions. The chip was scanned with the ArtixScan 120tf scanner (Micro TEK CO., USA) and the original data were analyzed using the attached software GEArrary analyzer. Each chip had 10 positive controls (2 for GAPDH, 4 for Ppia, 2 for RP113 and 2 for Actinb), three negative controls (PUC18DNA) and 3 blank controls. The original data were deduced by the background minimum value and then corrected by the content of home-keeping gene. The corrected data were analyzed for abundance of gene transcription between the two groups. The ratio $\geqslant 2$ was considered up-regulation of the gene and $\leqslant 0.5$ down-regulation[20].

Verification of IL-2 gene expression by RT-PCR

RNA extraction was done as previously described. The sample was RNA reverse transcripted to synthesized cDNA. The target gene and home-keeping gene of the sample were reacted by RT-PCR. A standard cure was plotted by measurement of the standard sample gradient to calculate the content of gene in the sample, which was corrected by the content of home-keeping gene to obtain the content of the related gene. All reagents used in the experiment were provided by Promega CO., USA. The sequences of β-actin (211 bp) and IL-2 (190 bp) are 5'-CCTGTACGCCAACACAGTGC-3' and 5'-ATACTCC TGCTTGCTGATCC-3', and 5'-CACTGACGCTTGTC CTCCTT-3' and 5'-TTCAATTCTGTGGCCTGCTT-3', respectively.

Statistical analysis

Data were represented as mean ± SD. SSPS 10.0 was used to perform t-test and F-test. $P < 0.05$ was considered statistically significant.

RESULTS

Mortality of rats after LPS injection and protective effect of 1,25-$(OH)_2D_3$

No death occurred in rats pretreated with 1,25-$(OH)_2D_3$ after LPS injection. Death occurred 9 h after LPS injection in rats pretreated with the vehicle, and the number of deaths was 5 within 24 h, with a mortality rate of 50%. There was no change in the number of deaths within 96 h.

Table 1 1,25-$(OH)_2D_3$-regulated LPS-induced cytokine production in rats (pg/mL, mean ± SD)

	1,25-$(OH)_2D_3$ + LPS (n = 15)	Vehicle + LPS (n = 15)	1,25-$(OH)_2D_3$ (n = 10)
IL-12	3986 ± 328[a]	4160 ± 289	69.99 ± 3.99[b]
IFN-γ	4840 ± 802[a]	5264 ± 524	5.42 ± 0.12[b]
IL-4	5.57 ± 1.75[a]	3.72 ± 1.60	

[a]$P < 0.05$ *vs* vehicle + LPS; [b]$P < 0.01$ *vs* 1,25-$(OH)_2D_3$ + LPS and vehicle + LPS.

1,25-$(OH)_2D_3$ inhibited LPS-stimulated production of IL-12 and IFN-γ in rats

Six hours after endotoxin stimulation, serum IL-12 and IFN-γ levels decreased significantly in rats pretreated with 1,25-$(OH)_2D_3$ as compared with those in rats pretreated with the vehicle. The serum level of these two cytokines was very low in rats not challenged by endotoxin, and there was a significant difference as compared with the previous two groups. As the serum IL-2 was below the limit of measurement in most rats 3 and 6 h after LPS attack, measurement was not done.

1,25-$(OH)_2D_3$ promoted IL-4 production in LPS-challenged rats

Six hours after endotoxin stimulation, serum IL-4 level elevated significantly in rats pretreated with 1,25-$(OH)_2D_3$ as compared with that in rats pre-treated with the vehicle. As the serum IL-4 was below the limit of measurement in most rats that are not attacked by LPS, measurement was not done (Table 1).

Quality control of RNA extraction

Electrophoresis showed that RNA extracted from the rat spleen displayed two clear bands (18S and 28S), and the absorbance at 260 nm and 280 nm was between 1.8 and 2.0, indicating that no RNA degradation occurred and the extract outcome was good.

1,25-$(OH)_2D_3$ regulated expression of Th1 and Th2 cytokines and related transcription factors

The gene chip used in the present experiment contains 95 target genes and other positive and negative controls. Expression difference was found in 39 genes between groups 1 and 2, accounting for 41% of the total number of the chip genes. These 39 genes include 10 up-regulated genes and 29 down-regulated genes (Table 2). The chip results showed that 1,25-$(OH)_2D_3$ down-regulated gene expression of Th1 and up-regulated gene expression of Th2, and the gene expression level in related transcription factors (Table 3).

Verification of down-regulation of IL-2 gene expression by RT-PCR

The results of the experiment showed that gene expression level in rats pretreated with 1,25-$(OH)_2D_3$ was significantly lower than that in rats pre-treated with the vehicle (0.476 ± 0.023 *vs* 0.678 ± 0.038, $P < 0.01$).

DISCUSSION

The purpose of the present experiment was to clarify the immune regulatory effect of 1,25-$(OH)_2D_3$ on Th1 dominant response *in vivo*. The results showed that 1,25-$(OH)_2D_3$ inhibited IFN-γ production of IL-12 and Th1 cytokines, suggesting that this inhibitory effect occurs at the transcription level. What implies in the results of the present experiment is the therapeutic effect of 1,25-$(OH)_2D_3$ on diseases mainly characterized by Th1 immune response (including autoimmune diseases) and transplantation rejection[16]. At the same time, as 1,25-$(OH)_2D_3$ affects the secretary profile of Th1 and Th2 cytokines[21,22], it inhibited the acute inflammatory reaction in the rats of group 1, indicating that 1,25-$(OH)_2D_3$ attenuates LPS lethal dose-induced injury in rats. The fact that all rats survived in group 1 suggests that 1,25-$(OH)_2D_3$ may also play a role in inhibiting the development and progression of acute inflammatory reaction.

IL-12 is a cytokine secreted by APCs and plays a central role in the growth of Th1 cells[23]. IL-12 has a potent biological function of inducing T cells to secrete IFN-γ[24]. IFN-γ is a pleiotropic cytokine, promoting inflammatory reaction and inducing expression of main tissue surface compatible complex of multiple cells[25]. Most recent studies found that this cytokine promotes vascular disease of the transplanted organ at the late stage of transplantation[26]. The present experiment confirmed that 1,25-$(OH)_2D_3$ could inhibited IL-12 production in rats, suggesting that it is able to inhibit strong Th1 immune response *via* its action on APCs, thus reducing IFN-γ production. At the same time, 1,25-$(OH)_2D_3$ may also directly inhibit the differentiation and proliferation of Th1 cells, as the cytokines mainly secreted by Th1 cells are reduced, especially transcription of NF-κB is inhibited. NF-κB is a key mediator of gene expression in immune and inflammatory responses. We also found that 1,25-$(OH)_2D_3$ inhibited proliferation of splenic lymphocytes in rats challenged with LPS. We, therefore, think that the results of the above experiment suggest that differentiation and proliferation of 1,25-$(OH)_2D_3$ on Th1 may also have an inhibitory effect on proliferation of splenic lymphocytes and is able to selectively inhibit Th1 immune response.

IL-4 is a main factor influencing the development of T cells into Th2 cells[13,27]. Once IL-4 level is able to resist activation of IL-12 on Th cells and IFN-γ on IL-4, it promotes differentiation of juvenile T cells to Th2 cells[28]. It is controversial over the regulatory effect of 1,25-$(OH)_2D_3$ on IL-4. It was reported that the effect of 1,25-$(OH)_2D_3$ is mediated through IL-4[5], and that it is the up-regulation of IL-4 and TGF-β by 1,25-$(OH)_2D_3$that inhibits the inflammatory reaction rather than by the reduction of Th1 cytokines IFN-γ and TNF-α[29]. It was also reported that 1,25-$(OH)_2D_3$ has no influence on the production of IL-4, or down-regulates IL-4[16,17]. We detected serum IL-4 levels in four batches of rats pretreated with 1,25-$(OH)_2D_3$ and

Table 2 Genes down-regulated by 1,25-$(OH)_2D_3$ in rat spleens

GenBank	Description	Gene name	Gene expression	Abundance	(Exp/vehicle)
NM007595	Calcium/calmodulin-dependent protein kinase Ⅱ, beta	CamK Ⅱ	0.00E + 00	0.00E + 00	0.00E + 00
NM009793	Calcium/calmodulin-dependent protein kinase Ⅳ	CamK Ⅳ	0.00E + 00	0.00E + 00	0.00E + 00
NM009843	Cytotoxic T-lymphocyte-associated protein 4	Cd152	9.20E - 02	0.00E + 00	0.00E + 00
NM031162	CD_3 antigen, zeta polypeptide	CD3Z antigen	5.00E - 02	0.00E + 00	0.00E + 00
NM007726	Cannabinoid receptor 1	cb1	0.00E + 00	3.12E - 02	0.00E + 00
NM009969	Colony stimulating factor	GM-CSF	1.88E - 01	0.00E + 00	1.38E - 01
NM022413	Nuclear factor of activated Epithelial calcium channel 2	Ecac 2	0.00E + 00	0.00E + 00	N/A
NM010118	Early growth response 2	Krox-20	2.99E - 02	0.00E + 00	N/A
NM010184	Fc receptor, IgE, high affinity 1, alpha polypeptide	Fcelα, Fcr-5	1.36E - 01	0.00E + 00	0.00E + 00
NM016863	FK506 binding protein 1b	FKBP 1B/FKBP	0.00E + 00	0.00E + 00	0.00E + 00
NM019827	Glycogen synthase kinase3 beta	Gsk-3	2.31E - 01	4.74E - 02	1.05E + 00
NM008284	Sarcoma virus oncogene 1	H-ras	0.00E + 00	0.00E + 00	1.03E - 02
NM008337	Interferon gamma	IFN-γ	3.44E - 01	1.24E - 02	1.02E + 00
NM008366	Interleukin 2	IL-2	3.98E - 01	2.79E - 01	2.26E - 01
NM008367	Interleukin 2 receptor, alpha chain	CD25	1.99E - 01	3.47E - 01	2.11E - 01
NM010591	Jun oncogene	c-JUN	0.00E + 00	0.00E + 00	0.00E + 00
NM019686	Kinase interacting protein 2	KIP 2	1.84E - 01	0.00E + 00	8.28E - 03
NM007746	Mitogen activated protein kinase 8	Cot	1.50E - 02	0.00E + 00	0.00E + 00
NM011951	Mitogen activated protein Mus musculus Harvey rat	P38MAPK	3.65E - 03	0.00E + 00	1.88E - 02
NM016700	Mitogen activated protein	JNK1	4.21E - 01	4.87E - 01	3.59E - 01
NM008656	Myogenic factor 5	Myf 5	1.83E - 02	0.00E + 00	6.37E - 01
NM016791	Nuclear factor of activated T-cell, cytoplasmic 1	NF-ATc	3.59E - 01	0.00E + 00	1.38E - 01
NM008915	Protein phosphatase 3, catalytic subunit, gamma isoform	Calcineurin A gamma	6.12E - 02	1.06E - 02	3.54E - 01
NM013693	Colony stimulating factor	GM-CSF	1.88E - 01	0.00E + 00	0.00E + 00
NM010188	Fc receptor, IgG, Low affinity Ⅲ	CD16	6.62E - 01	5.25E - 03	1.24E - 01
NM24684	Fos-like antigen 2	fra-2	5.59E - 01	0.00E + 00	0.00E + 00
NM41840	Protein phosphatase 3, regulatory subunit B, alpha isoform	Calcineurin B	2.23E + 00	4.01E - 01	4.73E - 01
NM010177	Tumor necrosis factor (ligand) superfamily, member 6	Fasl	1.02E + 00	0.00E + 00	1.88E - 01
NM019408	Nuclear factor of kappa light polyeptide gene enhancer in B-cell	NF-κB	4.58E - 01	3.50E - 01	4.25E - 01

N/A: Gene expression level, 1,25-$(OH)_2D_3$ = 0 and vehicle ⩾ 2.

Table 3 Genes up-regulated by 1,25-$(OH)_2D_3$ in rat spleens

GenBank	Description	Gene name	Gene expression	Abundance	(Exp/vehicle)
NM010548	Interleukin 10	IL-10	2.30E+00	8.25E-01	1.69E+00
NM010899	Nuclear factor of activated T-cell, cytoplasmic 2	NFAT1 (NFATP)	N/A	N/A	N/A
NM009192	Src-like adaptor	SLA	2.57E + 00	4.21E + 01	8.97E - 01
NM013672	Trans-acting transcription factor 1	Sp1	2.03E + 00	1.49E + 01	4.87E - 01
NM009505	Vascular endothelial growth factor A	VEGF/ VEGI	2.15E + 00	N/A	N/A
NM010234	FBJ osteosarcoma oncogene	c-fos	N/A	N/A	N/A
NM010510	Interferon beta, fibroblast	IFNb-1	N/A	N/A	N/A
NM010583	Mus musculus IL 2-inducible T-cell kinase	Tsk	6.77E - 01	N/A	5.29E + 00
NM021283	Interleukin 4	IL-4	N/A	N/A	N/A
NM010558	Interleukin 5	IL-5	N/A	N/A	N/A

N/A: Gene expression level, 1,25-$(OH)_2D_3$ ⩾ 2 and vehicle = 0.

those pretreated with the vehicle. Although we used inbreed line Lewis rats with little individual variance in establishing the model, we still found a significant individual difference in serum IL-4 level of the same experiment group, where the IL-4 level was lower than the test baseline in some rats. Only when we expanded the sample capacity, were the statistically significant results obtained. The results of gene chip test also showed that there was a great difference in IL-4 expression level between the rats 6 h after LPS stimulation. Only in one of the three rats in the study group, was IL-4 mRNA expression up-regulated by more than two times. However, as the capacity of the samples tested by gene chips was relatively small, and as there was still a tendency to up-regulate the gene expression of IL-4, IL-5 and IL-10 mainly secreted by Th2 cells, we performed another experiment, which confirmed again that 1,25-$(OH)_2D_3$ was able to up-regulate serum IL-10 level in rats challenged with LPS suggesting that 1,25-$(OH)_2D_3$ is able to promote the production of Th2 type cytokines[5,30], and at the same time inhibit the extent and progression of Th1 type immune response, forming the so-called "immune deviation" phenomenon[16], which is believed to help establish peripheral tolerance and is of significance in inhibiting transplantation rejection[4,31].

IL-12 is an allodiploid consisting of two subunits (P_{35} and P_{40}) encoded by two genes independently[32].

It is know that the P_{40} gene initiator region contains a NF-κB combining site[33]. The finding in the present experiment that 1,25-$(OH)_2D_3$ down-regulated the important transcription factor NF-κB, suggests that 1,25-$(OH)_2D_3$ reduces the expression of IL-12P40 subunit by inhibiting NF-κB, thus down-regulating assembly and secretion of IL-12 protein[33]. After activation of T cells, VDR is induced within 6 h, where IL-2 is the first expression-producing gene[34], and 1,25-$(OH)_2D_3$ inhibits the expression of IL-2 and IFN-γ mRNA, reaching the peak in 6-12 h[35]. It has been recognized that NF-κB and NF-ATp/c are specific transcription factors on IL-2 initiators[36,37]. It is also known that IFN-γ, GM-CSF, TNF-α, IL-4 and IL-5 initiators contain NF-AT element[34] and IL-4 enhancer contains 5 independent NF-AT sites, of which NF-ATp is a combining site of high affinity[34,38]. In the present experiment, increased IL-4 secretion by 1,25-$(OH)_2D_3$ might be related to up-regulation of NF-ATp. Although we were unable to identify the respective action of individual members of the NF-AT family on the expression of the cytokines in this study, we may still draw the conclusion that 1,25-$(OH)_2D_3$ influences the activity of NF-κB and NT-AT, two important transcription factors associated with cytokine regulation, by up-regulating NF-ATp gene expression and down-regulating NF-ATc gene expression. Both MAPK P38 and TNK pathways are mitocin-activated protein kinase pathways, not only closely associated with inflammatory reaction but also with cell growth, differentiation and apoptosis. 1,25-$(OH)_2D_3$ down regulates gene transcription of these important regulatory proteins in the MAPK pathways, suggesting that its influence on T help cell differentiation is the result of regulation on multiple signal pathways, and that the effector target of 1,25-$(OH)_2D_3$ regulating cytokines is at the gene transcription level.

COMMENTS

Background

1,25-dihydroxyvitamin D_3(1,25-$(OH)_2D_3$) has many effects on the production of cytokines, the gene expression maps of related cytokines and the mortality of rats. 1,25-$(OH)_2D_3$, the activated form of vitamin D, has, in addition to its central function in calcium and bone metabolism, important effects on the growth and differentiation of many cell types, and pronouced immunoregulatory properties. In the present study, the immunoregulatory effect of 1,25-$(OH)_2D_3$ in dominant Th1 response rats was investigated.

Research frontiers

Th1-biased immune response is associated with acute inflammatory conditions, and also plays a major role in a variety of human autoimmune diseases and graft rejection. However, few studies on the selective immunosuppression *in vivo* of 1,25-$(OH)_2D_3$ are available and the effect of 1,25-$(OH)_2D_3$ on gene expression profiles in Th cells is still unclear.

Innovations and breakthroughs

This is the first study to address the immunoregulatory effects of 1,25-$(OH)_2D_3$ in a dominant Th1 response model. The results show that 1,25-$(OH)_2D_3$ could regulate Th1-derived and Th2-derived cytokine production and protect rats from attacking of the LPS lethal dose.

Applications

The immunoregulatory properties of 1,25-$(OH)_2D_3$ were explored clinically for the topical treatment of psoriasis, a Th1 cell-mediated autoimmune disease of the skin. Our findings suggest that 1,25-$(OH)_2D_3$ may play an important role in Th1-inflammatory, autoimmune diseases and graft transplantation rejection.

Peer review

The manuscript "1,25-$(OH)_2D_3$ regulates LPS-induced cytokine production and reduces mortality in rats" by Qi XP *et al* presents experimental data from rats. The authors claim by pretreating rats with 1,25-$(OH)_2D_3$ that the LPS response is shifted towards a Th2-associated cytokine response with reduced Th1-associated cytokine response, so ensuring increased survival. The topic is of high interest.

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RAPID COMMUNICATION

Isolation and biological analysis of tumor stem cells from pancreatic adenocarcinoma

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Abstract

AIM: To explore the method of isolation and biological analysis of tumor stem cells from pancreatic adenocarcinoma cell line PANC-1.

METHODS: The PANC-1 cells were cultured in Dulbecco modified eagle medium F12 (1:1 volume) (DMEM-F12) supplemented with 20% fetal bovine serum (FBS). Subpopulation cells with properties of tumor stem cells were isolated from pancreatic adenocarcinoma cell line PANC-1 according to the cell surface markers CD44 and CD24 by flow cytometry. The proliferative capability of these cells *in vitro* were estimated by 3-[4,5-dimehyl-2-thiazolyl]-2, 5-diphenyl-2H-tetrazolium bromide (MTT) method. And the tumor growth of different subpopulation cells which were injected into the hypodermisof right and left armpit of nude mice was studied, and expression of CD44 and CD24 of the $CD44^+CD24^+$ cell-formed nodules and PANC-1 cells were detected by avidin-biotin-peroxidase complex (ABC) immunohistochemical staining.

RESULTS: The 5.1%-17.5% of sorted PANC-1 cells expressed the cell surface marker CD44, 57.8% -70.1% expressed CD24, only 2.1%-3.5% of cells were $CD44^+$ $CD24^+$. Compared with $CD44^-CD24^-$ cells, $CD44^+CD24^+$ cells had a lower growth rate *in vitro*. Implantation of 10^4 $CD44^-CD24^-$ cells in nude mice showed no evident tumor growth at wk 12. In contrast, large tumors were found in nude mice implanted with 10^3 $CD44^+CD24^+$ cells at wk 4 (2/8), a 20-fold increase in tumorigenic potential ($P < 0.05$ or $P < 0.01$). There was no obvious histological difference between the cells of the $CD44^+CD24^+$ cell-formed nodules and PANC-1 cells.

CONCLUSION: CD44 and CD24 may be used as the cell surface markers for isolation of pancreatic cancer stem cells from pancreatic adenocarcinoma cell line PANC-1. Subpopulation cells $CD44^+CD24^+$ have properties of tumor stem cells. Because cancer stem cells are thought to be responsible for tumor initiation and its recurrence after an initial response to chemotherapy, it may be a very promising target for new drug development.

Key words: Pancreatic tumor; Stem cells; Tumor stem cells; Isolation; Identification

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INTRODUCTION

Pancreatic carcinoma is an obstinate disease that is difficult to deal with. Though pancreatic cancer accounts for only 2%-3% of all cancers, it is the fourth most frequent cause of cancer death in industrialized countries[1]. It is estimated in the United States in 1998 that at least 29 000 new cases of pancreatic cancer will be diagnosed[2]. Unfortunately, only 18% will survive one year after diagnosis, the five-year survival rate is 4%. This is because by the time a patient exhibits symptoms, and the cancer is diagnosed, it is no longer in its early stage[3-5]. The main conventional treatments for pancreatic cancer are surgery, radiation therapy and chemotherapy. Despite advances in surgical and

medical therapy, little effect has been made on the mortality rate of this disease. According to Bjerkvig *et al*[6], the capacity of a tumor to grow and propagate is dependent on a small subset of cells (so-called tumor stem cells), tumor stem cells are immature cells that can replicate or self-renew, and are able to differentiate or grow into all the cells that an organism or particular organ system need. It has profound implications to understand how tumors evolve and how we treat tumors. If we can destroy these tumor stem cells, it will be possible to treat the patients successfully. However, it is difficult to purify tumor stem cells because of lack of specific cell surface markers in solid tumors. Recently, it was reported that cancer stem cells existed in some solid malignancies, including breast[7], brain[8,9], prostate[10], and lung cancers[11]. Thus, we deduced that pancreatic cancer might contain its own stem cells responsible for its metastasis and recurrence. To prove this hypothesis, we isolated subpopulation cells that have characteristics of tumor stem cells according to markers CD44 and CD24 by flow cytometry from pancreatic adenocarcinoma cell line PANC-1, and explore their biological characteristics. This study was to identify the method of isolation of pancreatic tumor stem cells and the ability of propagation of the tumor stem cells *in vitro* and *in vivo*.

MATERIALS AND METHODS

Experimental materials

Male nude mice, aged 6-8 wk and weighing 20 ± 2 g, were provided by the Experimental Animal Center, Hubei Center for Disease Control and Prevention, China. The nude mice were caged individually under specific pathogen free (SPF) conditions. Human pancreatic adenocarcinoma cell line PANC-1 was obtained from American Type Culture Collection, Manassas, Virginia, the Dulbecco modified eagle medium F12 (1:1 volume) (DMEM-F12) from Hyclone, Wuhan, China, the fetal bovine serum from Sijiqing, Hangzhou, China, trypsin from Sigma-Aldrich, Shanghai, China, the epidermal growth factor (EGF), basic fibroblast growth factor (b-FGF), insulin-transferrin-selenium solution (ITS) and trypsin from Sigma-Aldrich, Shanghai, China and PE anti-human CD44 and FITC anti-human CD24 were purchased from American Ancell.

Cell culture

The cells were cultured in incubator filled with 5% CO_2 at 37℃. The PANC-1 cells were cultured in DMEM-F12 (1:1 volume) supplemented with 20% fetal bovine serum (FBS), penicillin (1×10^5 U/L) and streptomycin (100 mg/L).

Flow cytometric analysis

Cells were dissociated by trypsin-EDTA solution (trypsin, 0.25%; EDTA, 0.02%) for 2-5 min at 37℃, transferred to a 5-mL tube, washed twice with PBS with 2% heat-inactivated calf serum (HICS; 5 min at 1000 r/min), resuspended in 100 μL (per 10^6 cells) of PBS, then were counted. PE anti-human CD44 and (or) FITC anti-human CD24 (appropriate dilution per antibody) were added and incubated for 30 min at 4℃, and then washed twice with PBS. Flow cytometry was performed on a FACS, and data were analyzed with the Cell Quest software (B.D., America). Using forward and side scatter profile, debris and dead cells were gated out. Cells were routinely sorted twice, and reanalyzed for purity. Then $CD44^+$, $CD44^-$ cells, $CD44^+CD24^+$ and $CD44^-CD24^-$ and unsorted cells were obtained.

Estimation of proliferative capability of cells in vitro

The $CD44^+CD24^+$, $CD44^-CD24^-$ and unsorted cells were diluted to a density of about 10^4 cells/mL with serum-free medium (SFM), a mixture of DMEM-F12 containing 10 ng/mL fibroblast and 20 ng/mL epidermal growth factors, 5 kg/mL insulin, 2.75 mg/mL transferrin, 2.75 ng/mL selenium (insulin-transferrin-selenium solution), penicillin (1×10^5 U/L) and streptomycin (100 mg/L). The 200-μL/well diluted cell suspension was plated to 96-well culture dishes. The wells with 2×10^3 cells were observed everyday under an Olympus CKX41 microscope; the images were captured using an Olympus C5050Z camera. Each group was set up with five duplicate holes. Their OD values were measured with spectrophotometer at 490 nm by 3-[4,5-dimehyl-2-thiazolyl]-2, 5-diphenyl-2H-tetrazolium bromide (MTT) method, and a 96-well plate was determined every 24h. The mean value was obtained and a growth curve was drawn.

Transplantation of cells into nude mice

After resuspension, $CD44^+$, $CD44^-$, $CD24^+$, $CD24^-$, $CD44^+CD24^+$, $CD44^-CD24^-$ and unsorted cells were diluted to a density of about 5×10^6 to 5×10^3 cells/mL with SCM. The cells (0.1 mL) were injected into the hypodermis of right and left armpit of nude mice. The mice were maintained in a specific pathogen-free room under constant temperature and humidity.

Immunohistochemical staining of CD44 and CD24

All samples of the $CD44^+CD24^+$ cell-formed nodules were placed into 10% formalin immediately, processed with routine histological procedures, and embedded in paraffin. Serial sections were cut 5 μm thick, and parts of them were stained with hematoxylin and eosin for routine histological observation under light microscope. The others were used for immunohistochemical examination for the CD44 and CD24. After deparaffinization (hydration), sections were treated sequentially with normal goat serum, anti-human CD44 polyclonal antibody (1:200) or anti-human CD24 polyclonal antibody (1:200), biotin-labeled goat anti-mouse IgG, and avidin-biotin-peroxidase complex (ABC). The sites of peroxidase binding were demonstrated by the diaminobenzidine method. Sections were then counterstained with hematoxylin for microscopic examination. Similar procedures were done for the PANC-1 cells. The numbers and areas of CD44-positive and CD24-positive foci > 0.2 mm in diameter and the total areas of the examined sections were measured using a Olympus C5050Z digital camera, Adobe Photoshop version 7.0, and Image-Pro Plus version 6.0.

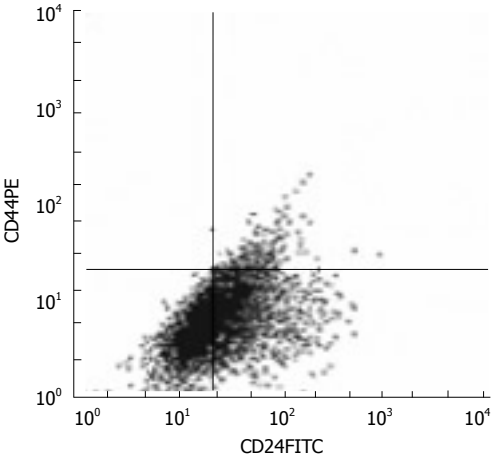

Figure 1 Analysis of Panc-1 pancreatic cancer cells by FACS.

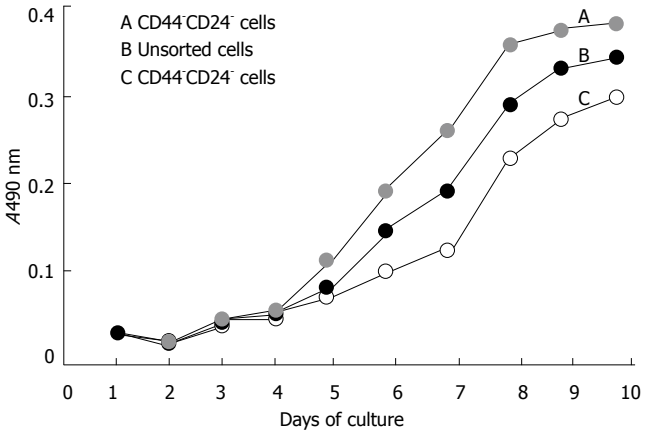

Figure 2 Growth curve of tumors cells *in vitro*.

Statistical analysis

Data were expressed as means ± SD, and were analyzed with SPSS 12.0, $P < 0.05$ was considered significant in difference.

RESULTS

Presence of CD44 and CD24 on cell surface of pancreatic carcinoma cell lines

To determine the presence of CD44 and CD24 on the cell surface of the PANC-1 cells, flow cytometric analysis was made. The cell surface markers CD44 and CD24 were chosen as a starting point based on prior work on breast cancer stem cells, in which $CD44^+CD24^{-/low}$Lineage tumorigenic cells generated tumors histologically similar to primary breast tumors when as few as 100 cells were transplanted, whereas tens of thousands of bulk unsorted cancer cells were needed to form tumors in NOD/SCID mice[7]. CD44 and CD24 have been identified as the stem cell surface markers, which act as adhesive molecules with multiple signaling functions[12-14]. As shown in Figure 1, 5.1%-17.5% of sorted PANC-1 cells expressed the cell surface marker CD44, and 57.8%-70.1% expressed CD24. When expression of multiple surface markers was examined, only 2.1%-3.5% of cells were $CD44^+$ $CD24^+$ (Figure 1).

Proliferation potential of cells in vitro

To evaluate the proliferation ability of cells *in vitro*, the $CD44^+CD24^+$, $CD44^-CD24^-$ and unsorted cells were cultured in SCM in 96-well culture dishes, their OD values were measured with spectrophotometer at 490nm by MTT method. Compared with $CD44^-CD24^-$ cells, $CD44^+CD24^+$ cells had a lower growth rate and longer doubling time *in vitro*. For the former, the index growth trend appeared at the 5th day, while the latter appeared at the day 7 (Figure 2).

Establishment of xenografts

To test the capability of tumor initiation, we injected cells into the hypodermis of right and left armpit of nude mice. When unsorted PANC-1 cells (5×10^3) were injected, no tumor growth was found at wk 12 while 10^4 cells were injected, one of six mice developed tumors. For cancer cells sorted for the markers CD44 and CD24, expression of individual markers identified cell populations with enhanced tumorigenic potential. For example, injection of 5×10^3 $CD44^+$ cells would occasionally form a tumor (1 of 6 animals), whereas no tumor was observed with $CD44^-$ cells until at least 5×10^4 cells were injected (1 of 10 animals). Six of 10 animals developed tumors when injected with 5×10^4 $CD44^+$ cells, representing a 10-fold increase in tumorigenic potential compared with marker negative cells ($P = 0.029$). Similar results were obtained with $CD24^+$. Injection of $CD44^+CD24^+$ cells resulted in an enhanced tumorigenic potential compared with single marker sorted cells. More tumors formed with injection of as few as 10^3 cells, and no tumor formed in marker-negative cells until at least 5×10^4 cells were injected. The sorted cell population with the highest tumorigenic potential was those expressing CD44 and CD24. For example, injection of 10^4 $CD44^-$ $CD24^-$ cells in nude mice found no tumor growth at wk 12. In contrast, nude mice injected with 10^3 $CD44^+CD24^+$ cells had large tumors at wk 4 (2 of 8), a 20-fold increase in tumorigenic potential ($P < 0.05$ or $P < 0.01$) (Table 1). There was no obvious histological difference between the $CD44^+CD24^+$ cell-formed nodules and PANC-1 cells.

$CD44^-$ and $CD24^-$ positive numbers and areas

For sections stained with hematoxylin and eosin, tumor cells with variable shape from polygon, spindle to irregular were seen under light microscope. The total $CD44^-$ positive and $CD24^-$ positive numbers and areas in the examined sections were measured using an Olympus C5050Z digital camera, Adobe Photoshop version 7.0, and Image-Pro Plus version 6.0. There was no significant difference in quantitative values of $CD44^+$ and $CD24^+$ cells between the formed nodules and the PANC-1 cells ($P > 0.05$) (Figure 3).

DISCUSSION

The theory of tumor stem cells[15,16] indicates that tumor cells have heterogeneity, i.e., the majority of cells in the

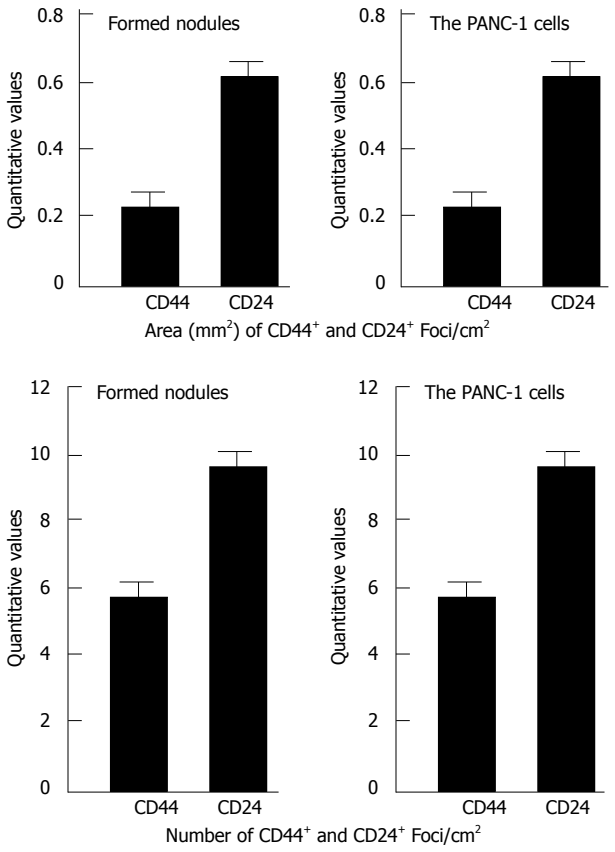

Figure 3 Quantitative values of $CD44^+$ and $CD24^+$ cell foci in the formed nodules and PANC-1 cells. There was no significant difference between the formed nodules and PANC-1 cells.

tumor have lost the growth potential, only a small subset of cells have the capability of the infinite proliferation, the differentiation and the formation of cloning *in vitro*. The initial isolation and identification of tumor stem cells was first proved in hematological malignancies. The $CD34^+CD38^-$ phenotype cells (5% of the cancer cells) with obvious proliferation, differentiation and self-renewal ability were purified from the blood of the patients with acute myeloid leukemia[17,18]. In 2003, researchers found that only a small subset of human breast cancer cells, with the phenotype $CD44^+CD24^-$, formed new tumors in NOD/SCID mice[7]. These breast cancer-initiating cells can be isolated and propagated *in vitro* as extensively proliferating, clonal, nonadherent spherical clusters are able to differentiate along different mammary epithelial lineages[19]. A small population of cancer-initiating cells (also called cancer stem cells) was later found in several malignancies, including brain[8], prostate[10], liver[20,21], lung[22], melanoma[23], and colon tumors[24,25].

Although there is increasing evidence that a rare population of undifferentiated cells is responsible for tumor formation and maintenance, little work has been done on the identification of pancreatic cancer special surface markers or on isolation of pancreatic tumor-initiating cells. Based on studies in breast cancer[7] and pancreatic adenocarcinoma[16], we identified cells with the characteristics of tumor stem cells according to the cell surface markers CD44 and CD24 by flow cytometry from

Table 1 Tumor formation ability of sorted pancreatic cancer cells using surface markers (number of tumors formed/number of injections)

Groups	5×10^5	10^5	5×10^4	10^4	5×10^3	10^3	10^2
Unsorted	6/6	5/6	3/6	1/6	0/6	0/0	0/0
$CD44^+$	0/0	9/10	6/10	3/10	1/6	0/4	0/0
CD44	0/0	2/10	1/10	0/4	0/0	0/0	0/0
P		0.0027	0.0286	0.3297			
$CD24^+$	0/0	7/8	4/8	3/8	0/4	0/0	0/0
CD24	0/0	2/8	1/8	0/8	0/0	0/0	0/0
P		0.0203	0.1410	0.1000			
$CD44^+$ $CD24^+$	0/0	8/8	7/8	6/8	4/8	2/8	0/4
CD44 CD24	0/0	1/8	1/8	0/8	0/8	0/8	0/0
P		0.0007	0.0051	0.0035	0.0385	0.2333	

Compared with results from marker-negative cells.

pancreatic adenocarcinoma cell line PANC-1. Tumor stem cells have the capability to maintain themselves in culture in an undifferentiated state, initiate tumor growth after xenotransplantation in mice, and differentiate into cancers that are phenotypically indistinguishable from the original tumor. We found that 5.1%-17.5% of sorted PANC-1 cells expressed the cell surface marker CD44, 57.8%-70.1% expressed CD24, and only 2.1%-3.5% of cells were $CD44^+CD24^+$. To take a small subset of cells and put it in the organism and see if it regenerates the original tissues is the classic definition of a stem cell. We injected cells into the hypodermis of the right and left armpit of nude mice to test the capability of tumor initiation. When 5×10^3 unsorted PANC-1 cells were injected into nude mice, no tumor grew at wk 12 unless at least 10^4 cells were injected. For cancer cells sorted for the markers CD44 and CD24, injection of 5×10^3 $CD44^+$ cells would form a tumor, whereas no tumor was observed with $CD44^-$ cells until at least 5×10^4 cells were injected. Similar results were obtained with $CD24^+$. The sorted cell population with the highest tumorigenic potential was those cells expressing CD44 and CD24. For instance, injection of 10^4 $CD44^-CD24^-$ cells into nude mice, no tumor growth was evident at wk 12. In contrast, nude mice injected with 10^3 $CD44^+CD24^+$ cells had large tumors at wk 4. Moreover, the $CD44^+CD24^+$ cells maintained the ability to engraft and reproduce the same histological and antigenic pattern of the PANC-1. In addition, compared with $CD44^-CD24^-$ cells *in vitro*, $CD44^+CD24^+$ cells had a lower growth rate. The reason is that tumor stem cells are similar to stem cells, which is in relatively static group of cells, and besides other primates, the stem cell pool proliferates once a year[26]. For the $CD44^+CD24^+$ cells, there were biological behaviors of the lower proliferative index and the faster tumor growth rate *in vivo*. It is self-contradictory. The reason awaits further studies. In addition, there was no obvious histological difference between the $CD44^+CD24^+$ cell-formed nodules and PANC-1 cells.

The above results showed that CD44 and CD24 may be used as markers for isolation of pancreatic cancer stem cells from pancreatic adenocarcinoma cell line PANC-1, subpopulation cells $CD44^+CD24^+$ have the characteristics of tumor stem cells. The purification and

other biological behaviors of pancreatic adenocarcinoma stem cells need to be further studied in the future.

COMMENTS

Background

Pancreatic carcinoma is an obstinate disease that is difficult to deal with. Though pancreatic cancer accounts for only 2%-3% of all cancers, it is the fourth most frequent cause of cancer deaths in industrialized countries. Unfortunately, only 18% will survive one year after diagnosis, the five-year survival rate is only 4%. Conventional main treatments for pancreatic cancer are surgery, radiation therapy and chemotherapy. Despite advances in surgical and medical therapy, little effect has been achieved on the mortality rate of this disease.

Research frontiers

The initial isolation and identification of tumor stem cells was first proved in hematological malignancies. The $CD34^+CD38^-$ phenotype cells (5% of the cancer cells) with obvious proliferation, differentiation and self-renewal ability had been purified from the blood of the patients with acute myeloid leukemia. Researchers have discovered a small population of cancer-initiating cells (also called cancer stem cells) in several malignancies, including brain, prostate, liver, lung, melanoma, and colon tumors.

Innovations and breakthroughs

The authors isolated pancreatic adenocarcinoma cell line PANC-1 according to the cell surface markers CD44 and CD24 by flow cytometry, obtained subpopulation cells which have properties of tumor stem cells, and identified the ability of propagation of the tumor stem cells *in vitro* and *in vivo*.

Applications

Because cancer stem cells are thought to be responsible for tumor initiation and its recurrence after an initial response to chemotherapy, it may be a very promising target for new drug development.

Peer review

This study corroborates a recent publication in the pancreas reporting that a subpopulation of Panc1 cells can propagate to form spheres and that these cells express stem cell markers such as CD44. The study is very interesting.

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RAPID COMMUNICATION

Assessment of hepatic VX_2 tumors with combined percutaneous transhepatic lymphosonography and contrast-enhanced ultrasonographic imaging

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Abstract

AIM: To evaluate the feasibility and efficacy of percutaneous transhepatic lymphosonography (PTL) as a novel method for the detection of tumor lymphangiogenesis in hepatic VX_2 of rabbits and to evaluate combined PTL and routine contrast-enhanced ultrasonographic imaging for the diagnosis of liver cancer.

METHODS: Ten rabbits with VX_2 tumor were included in this study. SonoVue (0.1 mL/kg) was injected into each rabbit *via* an ear vein for contrast-enhanced ultrasonographic imaging, and 0.5 mL SonoVue was injected into the normal liver parenchyma near the VX_2 tumor for PTL. Images and/or movie clips were stored for further analysis.

RESULTS: Ultrasonographic imaging showed VX_2 tumors ranging 5-19 mm in the liver of rabbits. The VX_2 tumor was hyperechoic and hypoechoic to liver parenchyma at the early and later phase, respectively. The hepatic lymph vessels were visualized immediately after injection of contrast medium and continuously visualized with SonoVue® during PTL. The boundaries of VX_2 tumors were hyperechoic to liver parenchyma and the tumors. There was a significant difference in the values for the boundaries of VX_2 tumors after injection compared with the liver normal parenchyma and the tumor parenchyma during PTL.

CONCLUSION: PTL is a novel method for the detection of tumor lymphangiogenesis in hepatic VX_2 of rabbits. Combined PTL and contrast-enhanced ultrasonographic imaging can improve the diagnosis of liver cancer.

Key words: Percutaneous transhepatic lymphosonography; Ultrasound; Contrast-enhanced ultrasonographic imaging; Ultrasound contrast media; VX_2 tumor

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INTRODUCTION

The liver is the largest organ in the abdominal cavity and the main region of primary tumor and distant metastasis of malignant tumors. Detection of tumor nodules in the liver is of major importance for formulating therapeutic strategies and predicting the prognosis in malignant tumors[1].

Non-ionizing radiation, portable and noninvasive real-time imaging[2,3], ultrasonography (US) are the most commonly used imaging techniques. Introduction of microbubbles as contrast agents for ultrasound has improved the image quality and diagnostic value[4-8]. Contrast-enhanced ultrasonographic imaging enables noninvasive measurements of microvascular perfusion in the heart, brain, kidney, skeletal muscle, skin grafts and solid tumors[9] and provides functional images of angiogenesis in animals and humans.

At present, contrast-enhanced ultrasonographic imaging research has mainly focused on angiogenic blood vessels, blood vessel function and efficacy of

angiogenesis inhibitors. Recently, lymphangiogenesis has become a new research frontier[10]. Tumor lymphangiogenesis is the process of forming new lymph vessels in tumors and closely related to tumor development and progression. It is necessary to find noninvasive methods for evaluating lymphangiogenesis *in situ*. However, little is known about the contrast-enhanced ultrasonographic imaging used to detect tumor lymphangiogenesis. Recently, lymphosonography after interstitial injection of microbubble-based contrast agents can trace the lymphatic channels from the injection site up to the draining sentinel lymph nodes[11-15]. However, no report is available on lymphosonography for tumor lymphangiogenesis.

The aim of the present study was to evaluate the feasibility and efficacy of PTL with a small volume of SonoVue® as a novel method for the detection of tumor lymphangiogenesis of hepatic VX_2 in rabbits and to evaluate the combined PTL and contrast-enhanced ultrasonographic imaging in the diagnosis of liver cancer.

MATERIALS AND METHODS

Animal model

Ten male health New Zealand rabbits, weighing 2.5-3.0 kg, were included in this study and housed in an approved facility with free access to water and standard diet throughout the study. The study, approved by the Institutional Review Board for Animal Research, was performed following the Guidelines for the Care and Use of Laboratory Animals[16].

An undifferentiated VX_2 carcinoma growing rapidly in rabbits served as the experimental tumor. Two VX_2 tumors were implanted into the right and left lobes of liver, respectively. In brief, rabbits were anesthetized with ketamine hydrochloride (40 mg/kg) and xylazine hydrochloride (5 mg/kg) intramuscularly. The rabbits were intermittently given small supplementary doses of sodium pentobarbital (ranging from 3.1 to 6.5 mg/kg) during the experiment to maintain adequate sedation, fixed in a supine position on a rigid board of paper. Hair on the abdominal skin was shaved after the animals became stable. Diagnostic US was performed to assess the implantation site. Cryoconserved tumor material, implanted in the lower leg muscles of an additional animal and harvested after it reached a size of 1.5 cm, was placed into a saline solution and cut into sections measuring 1 mm × 1 mm × 1 mm.

The implantation method used has been described elsewhere[17]. Only part of tumor tissue showing no macroscopic signs of necrosis was used. A 16-gauge intravenous cannula was placed into the left and right liver lobe respectively under US guidance, and the prepared tumor tissue sections were pushed through the cannula and placed at the preselected position. The same procedure was performed on each animal. The rabbits were permitted to recover and followed up sonographically (Sequia 512, Siemens, Germany) weekly until a localized, avascular carcinoma-like mass developed at the injection site after 10-15 d.

Equipment

Sequia 512 US image system was purchased from Siemens, Germany, with a L15-8 probe equipped for Cadence CPS software. Its acoustic output was carefully controlled by the operator. MI was set at 0.1-0.3 in order to avoid considerable bubble destruction and reduction of the contrast effect. Cadence CPS is a real-time, non-linear imaging technique specific for the second echo-contrast agent examination. Cadence CPS processing utilizes all non-linear responses, fundamental and higher order harmonics, to produce high sensitivity contrast agent images with excellent agent-to-tissue specificity at a very low MI. Images and/or movie clips were stored during PTL and contrast-enhanced ultrasonographic imaging.

Contrast agent

Contrast agent used in this study was SonoVue® (Bracco, Milan, Italy). Microbubbles are sulfur hexafluoride stabilized in a phospholipid shell, 1-10 μm in diameter, averaging about 2.5 μm. The SonoVue® preparation was reconstituted just before administration by adding 5 mL sterile saline to the freeze-dried powder, so that sulfur hexafluoride had a concentration of 45 μg/mL in the suspension.

SonoVue® injection

SonoVue (0.1 mL/kg) was injected *via* an ear vein as a rapidly injected bolus, followed by a 1.5 mL saline flush for routinely contrast-enhanced ultrasonographic imaging.

SononVue (0.5 mL) was injected into the normal liver parenchyma near the VX_2 tumors as a rapidly injected bolus using a tuberculin syringe and a 26-gauge needle for PTL. The absorption of the contrast agent and its flow were observed in lymphatic channels of the VX_2 tumors.

Statistical analysis

For quantitative analysis, videodensities of the appropriate regions of interest (ROI), including perineoplastic liver parenchyma, boundaries of the tumor and tumor parenchyma were recorded during PTL. Respective evaluations were made for PTL. Data analysis was carried out using SPSS 16 statistical software. All videodensity data were expressed as mean ± SD. Parameters were tested using paired *t* test. Statistical analysis was performed using one-way analysis of variance and Dunnett's multiple comparison tests. $P < 0.05$ was considered statistically significant.

RESULTS

The VX_2 tumor in liver of rabbits ranging 5-19 mm was found to be a low echoic mass. However, because the VX_2 tumor was almost isoechoic with the normal tissue and boundaries of the masses were unclear, detection and delineation of the lesion were difficult before SonoVue® injection (Figure 1A).

Since the typical enhancement pattern of VX_2 tumor detected by routine contrast-enhanced ultrasonographic imaging was hyperechoic and hypoechoic to liver

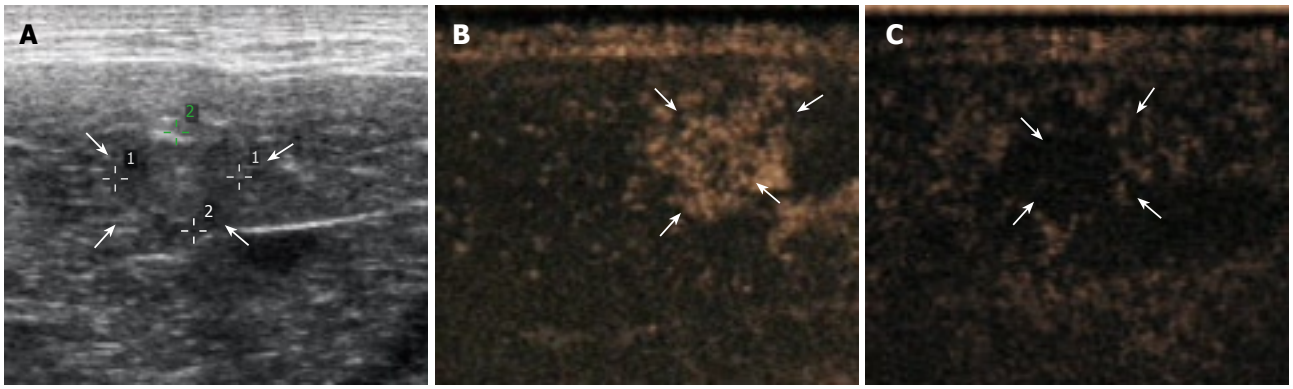

Figure 1 Liver of a VX_2 tumor-bearing rabbit imaged in the conventional mode before (**A**), immediately after 18 s (**B**) and 96 s (**C**) of injection of 0.1 mL sonazoid microbubbles/kg. Arrows indicate VX_2 tumor.

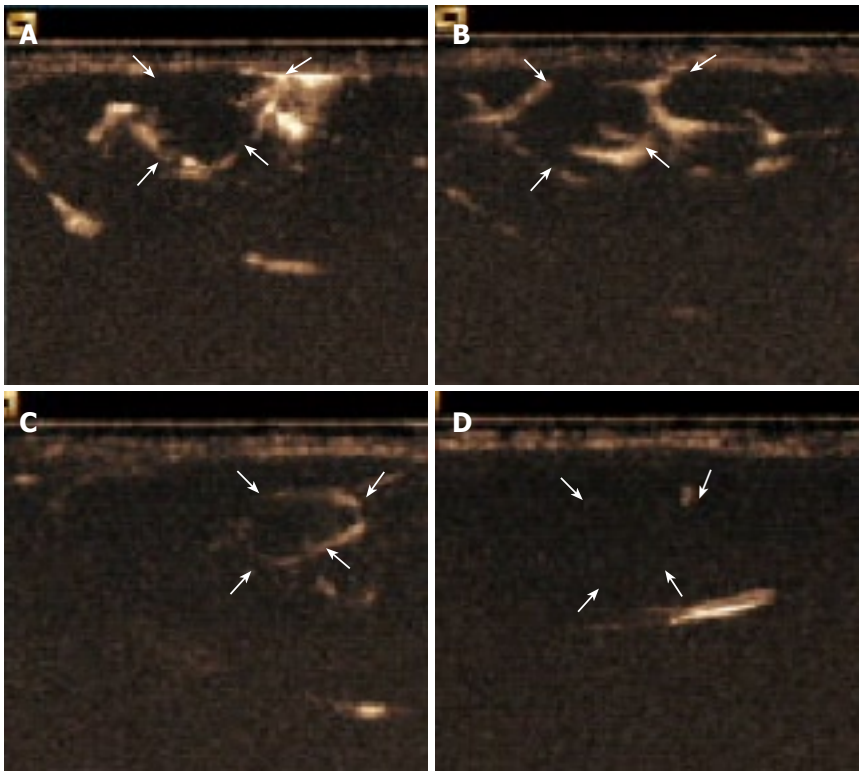

Figure 2 Hepatic lymph vessels visualized 36 s (**A**), 4 min (**B**), 7 min (**C**) 18 min (**D**) after injection of contrast agent and continuously visualized with SonoVue® during PTL with hyperechoic boundaries of VX2 tumors to liver parenchyma and the tumor.

parenchyma during the early and later phase, respectively, a much more rapid wash-in and -out of ultrasonographic contrast agent was observed compared to the normal liver parenchyma (Figure 1B and C).

The enhancement pattern of VX_2 tumors detected by PTL was significantly different from the typical enhancement pattern of VX_2 tumors detected by routine contrast-enhanced ultrasonographic imaging. The hepatic lymph vessels were visualized immediately and continuously during PTL. SonoVue® was deposited in the parenchyma relatively quickly in winding channels. At the same time, the boundaries of VX_2 tumors were hyperechoic to liver parenchyma and the tumors. The hyperechoic boundaries clearly delineated VX_2 tumors compared with the normal liver and tumor parenchyma (Figure 2A-C). The difference in the videodensitometric measurements of the boundaries of VX_2 tumors was significantly higher than the baseline (Figure 3). Conversely, videodensity in the normal liver and tumor parenchyma had no signal enhancement compared with the baseline (Figure 3). There was a significant difference in the boundaries of VX_2 tumors compared with the baseline as well as the normal liver and tumor parenchyma (Figure 3).

DISCUSSION

Ultrasound is an important and useful imaging method for the detection of tumors. Ultrasound contrast agents containing encapsulated microbubbles are mainly used to increase the diagnostic imaging of tumors. McCarville *et al*[18] showed that gray-scale US measurements of microbubble contrast agent flow can be used to detect the functional consequences of antiangiogenic therapy for tumors and to assess angiogenesis inhibitors that act through different mechanisms[19-23].

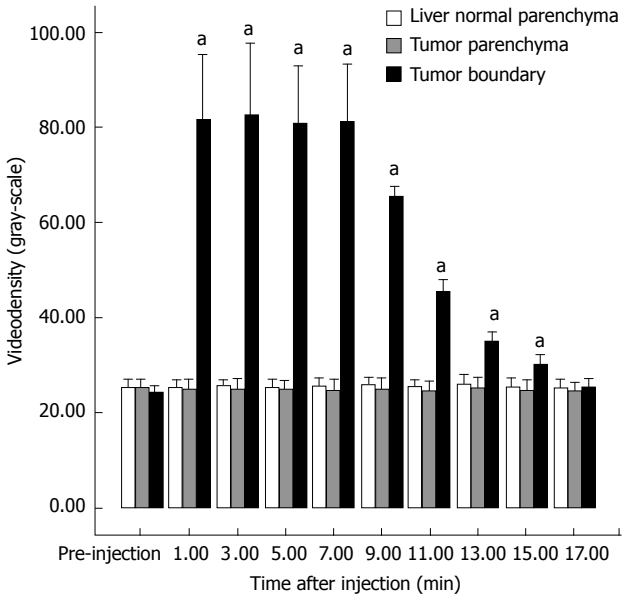

Figure 3 Videodensitometric measurements of liver normal parenchyma (white), tumor parenchyma (gray) and tumor boundary (black) before and after percutaneous transhepatic injection of contrast agent SonoVue® into the normal liver parenchyma near the VX_2 tumors during PTL. $^aP < 0.05$ *vs* respective pre-injection values (Dunnett).

Recently, lymphangiogenesis has become a new research frontier[10]. The important functions of the lymphatic system are to remove damaged cells from the body and to prevent the spread of infection and cancer for the maintenance of normal tissue fluid balance and immune surveillance. In spite of its important functions in physiological and pathological conditions, including tumor metastasis, lymphoedema and inflammation, lymphatic vessels have not received as much attention as blood vessels, and the mechanisms regulating their development and growth have been poorly understood[24]. Lymphangiogenesis is associated with increased tumor cells in lymphatics and lymph nodes, served as an independent prognostic factor and a potential target in the development of new therapies for hilar cholangiocarcinoma[25]. At present, neovessel formation, including lymphangiogenesis, represents the key event in tumor progression. Inhibition of metastatic spread may be achieved by restriction of lymphatic vessel growth with novel therapeutic strategies for anti-lymphangiogenic therapies[26].

Currently, histologic determination of the mean intratumoral or peritumoral lymphatic vessels is the most commonly used method for assessing lymphangiogenesis. However, obtaining tissue for histologic evaluation may require an invasive procedure that cannot be normally accepted by patients. Furthermore, determination of the lymphatic microvessel density does not provide an accurate assessment of the functionality of tumor lymphatic vessels because many poorly functioning or collapsed lymphatic vessels have endothelial cells that are stained and counted. Therefore, the lymphatic microvessel density *in vivo* may be a potentially useful marker for assessing lymphangiogenesis in tumors at diagnosis, and accurately reflects the effectiveness of antitumor therapy.

Ultrasound lymphography with subcutaneous injection of ultrasound contrast material enables direct visualization of the lymphatic drainage pathways and sentinel lymph nodes of breast diseases, melanoma, *etc*[11-15].

In the present study, the traditional percutaneous hepatic injection method was used to deliver SonoVue® microbubbles into the liver under US guidance to investigate tumor lymphangiogenesis. To the best of our knowledge, lymphosonography for the detection of tumor lymphangiogenesis has not been reported before. Hepatic lymph vessels were visualized immediately after injection of contrast agent and opacified with SonoVue® during PTL, whereas liver parenchyma was not enhanced by SonoVue®. SonoVue® was deposited in the parenchyma relatively quickly in winding lymph vessels. At the same time, the boundaries of VX_2 tumors were hyperechoic to liver parenchyma and the tumors, indicating that hyperechoic boundaries clearly delineate the peritumoral lympathatic vessels of VX_2 tumors. Compared with the hyperechoic boundaries of VX_2 tumors, the videodensity in the tumor parenchyma had no signal enhancement compared with the baseline. This is consistent with the findings in a previous study[27]. It was reported that three-dimensional changes of lymphatic architecture in rabbit VX_2 tongue cancer, dynamics of its adjacent lymphatic architecture, especially the increased number of capillaries in preexisting lymphatic vessels outside the tumor margin, are associated with lymph node metastasis[28,29]. The morphological features of lymphatic vessels during PTL may be important predictive markers for evaluating lymphatic metastasis and prognosis of tumors. The lymphatic drainage paths and lymphatic distribution pattern in hepatic tissue have been found to be very constant, showing that angiogenesis is a critical factor for tumor growth and metastasis[23]. In this study, the typical enhancement pattern of VX_2 tumors detected by routine contrast-enhanced ultrasonographic imaging was hyperechoic and hypoechoic to the liver parenchyma at the early and later phases, respectively, confirming that routine contrast-enhanced ultrasonographic imaging can assess tumor vascularity and reveal the microvascular perfusion and function[23,30,31].

The specific mechanism by which the contrast agents used in this study enter the lymphatic system is unclear. SonoVue® microbubbles have a mean diameter of 2.5 μm with 99% smaller than 11 μm, allowing a free passage of capillaries, but keeping within the vascular lumen. This means that SonoVue® microbubbles in the hepatic inter-space cannot come into blood vessels. Although the optimal particle diameter for lymphatic uptake is 10-50 nm, particles up to hundreds of nanometers in diameter appear to be able to cross the lymphatic endothelium[32-34]. Due to the flexibility of microbubbles, phospholipidic shell and poor solubility and diffusivity of SF_6, SonoVue® is highly resistant to pressure. This means the microbubbles may more easily distort and traverse lymphatic wall fenestrations into lymph capillaries.

Due to the different membranes, 99% of Sonazoid and Optison are phagocytosed by Kupffer cells, whereas only 7.3% of SonoVue® is phagocytosed by Kupffer cells[35]. This means that the SonVue® microbubbles are

not easily phagocytosised by macrophages. Tracing the SonVue® microbubble flowing in the lymph vessels can improve the pathologic staging of the disease and its treatment.

At the same time, microbubbles are used not only for contrast enhancement of ultrasound images and improvement of diagnosis, but also for delivery of drugs and genes[36-40]. The ability to localize lymphatic vessels in tumors may be of value for a new route to the administration of drugs, gene and immunotherapy, *etc*. Drugs/genes containing vesicles may be injected simultaneously with microbubbles or microbubbles in combination with microbubble-forming vesicle aggregates. Using microbubbles oscillation and cavitation under US guidance might assist in delivering drugs/genes from vesicles to the interstitial tissue, which may be an effective treatment for some diseases.

Since few studies about hepatic lymphography are available at present, it is difficult to find microbubbles in lymphatic vessels. Due to this reason, the study only limited to the ultrasound characteristic aspects of PTL, which were not compared with the histopathologically aspects of rabbit VX_2 tumors.

In conclusion, PTL with a small volume of SonVue microbubbles is a novel method for the detection of tumor lymphangiogenesis of hepatic VX_2 in rabbits. Combined PTL and contrast-enhanced ultrasonographic imaging can improve the diagnosis of liver cancer. Additional research is needed to determine the potential advantages of PTL and to determine if PTL can be used in clinical practice.

COMMENTS

Background

Ultrasonography (US) is one of the most commonly used imaging techniques. Lymphangiogenesis has become a new research frontier. Tumor lymphangiogenesis is the process of generating new lymph vessels within and surrounding tumors, which is closely related to tumor development and progression. It is neccessary to develop noninvasive methods for evaluating lymphangiogenesis *in situ*. However, to the best of our knowledge, lymphosonography showing tumor lymphangiogenesis with percutaneous hepatic injection of ultrasound contrast material has not been reported before.

Research frontiers

This study investigated tumor angiogenesis and lymphangiogenesis with combined percutaneous transhepatic lymphosonography (PTL) and contrast-enhanced ultrasonographic imaging for hepatic VX_2 in rabbit liver.

Innovations and breakthroughs

Contrast-enhanced ultrasonographic imaging enables noninvasive measurements of microvascular perfusion in the heart, brain, kidney, skeletal muscle, skin grafts and solid tumors in animals and humans. It was recently reported that lymphosonography after interstitial injection of microbubble-based contrast agents can trace lymphatic channels from the injection site up to the draining sentinel lymph nodes. This is the first study to evaluate the feasibility and efficacy of PTL with a small volume of SonoVue® as a novel method for the detection of tumor lymphangiogenesis of hepatic VX_2 in rabbits and to evaluate the role of combined PTL and contrast-enhanced ultrasonographic imaging in improving the diagnosis of liver cancer.

Applications

PTL with a small volume of SonVue microbubbles is a novel method for the detection of tumor lymph angiogenesis of hepatic VX_2 in rabbits. Combined PTL and contrast-enhanced ultrasonographic imaging can improve the diagnosis of liver cancer. Additional research is needed to determine the potential advantages of PTL and to determine if PTL can be used in clinical practice.

Peer review

PTL is a new tool for the diagnosis of liver cancer. The study is well designed and interesting.

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RAPID COMMUNICATION

Tuberculosis *versus* non-Hodgkin's lymphomas involving small bowel mesentery: Evaluation with contrast-enhanced computed tomography

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Dong P, Wang B, Sun QY, Cui H. Tuberculosis *versus* non-Hodgkin's lymphomas involving small bowel mesentery: Evaluation with contrast-enhanced computed tomography. *World J Gastroenterol* 2008; 14(24): 3914-3918 Available from: URL: http://www.wjgnet.com/1007-9327/14/3914.asp DOI: http://dx.doi.org/10.3748/wjg.14.3914

Abstract

AIM: To evaluate the specific computed tomography (CT) imaging criteria for differentiating tuberculosis involving the small bowel mesenteric lymph nodes from lymphomas.

METHODS: We retrospectively reviewed the anatomic distribution, CT enhancement patterns of lymphoma in 18 patients with mesenteric tuberculosis and 22 with untreated non-Hodgkin's lymphomas (NHL) involving small bowel mesentery (SBM). Of the 18 patients with tuberculosis, 9 had purely mesenteric tuberculous lymphadenopathy (TL), and 9 had mesenteric TL accompanied with tuberculous mesenteritis (TLM).

RESULTS: CT showed that tuberculosis and NHL mainly affected lymph nodes in the body and root of SBM. Homogeneously enhanced lymph nodes in the body and root of SBM were found more often in the NHL ($P < 0.05$). Homogeneously mixed peripheral enhanced lymph nodes in the body of SBM were found more often in mesenteric TL and TLM ($P < 0.05$). Peripheral enhanced lymph nodes in the root of SBM were found more often in mesenteric TL and TLM ($P < 0.01$). "Sandwich sign" in the root of SBM was observed more often in NHL ($P < 0.05$).

CONCLUSION: Anatomic lymph node distribution, sandwich sign and specific enhancement patterns of lymphadenopathy in SBM on CT images can be used in differentiating between tuberculosis and untreated NHL involving SBM.

INTRODUCTION

The incidence of tuberculosis is increasing[1-4]. Abdominal tuberculosis can affect the gastrointestinal tract, peritoneum and lymph nodes. When the prevalence of abdominal tuberculosis is high, it is difficult to establish its diagnosis[5-7]. Lymphadenopathy is the most common manifestation of abdominal tuberculosis and may be easily confused with lymphomas involving abdominal lymph nodes in up to 55% of cases without other evidence of abdominal involvement[8]. Clinical and radiologic differentiation between the two can be challenging[6,8,9]. To our knowledge, a comparison of computed tomography (CT) findings in tuberculosis and lymphoma of the mesenteric lymph nodes has not been reported[10]. Lymphoma[11] is the most common malignant neoplasm affecting the mesentery and Hodgkin's Lymphoma can rarely involve the mesentery[10], so we conducted a comparison of CT findings in tuberculosis and non-Hodgkin's lymphoma (NHL) involving the small bowel mesentery (SBM) to improve the physicians' ability to distinguish between these entities.

MATERIALS AND METHODS

We retrospectively reviewed the medical records of 40 consecutive patients with documented tuberculosis [18 (45%) with mesenteric tuberculosis and 22 (55%) with untreated non-Hodgkin's lymphomas (NHL) involving small bowel mesentery (SBM)] who underwent contrast-

Table 1 Anatomic distribution and enhancement patterns in mesenteric lymph nodes

	TL (n = 9)			TLM (n = 9)			NHL (n = 22)		
	Margin of SBM	Body of SBM	Root of SBM	Margin of SBM	Body of SBM	Root of SBM	Margin of SBM	Body of SBM	Root of SBM
Homogeneous	0	2	3	0	5	5	5	20	18
Peripheral	1	2	6	0	0	4	0	0	0
Homogeneously mixed peripheral	0	4	0	0	4	0	0	2	4

TL: Tuberculous lymphadenopathy; TLM: Tuberculous lymphadenopathy accompanied with mesenteritis; NHL: Non-Hodgkin's lymphoma.

enhanced CT from October 1998 to May 2007 in our hospital. The patients with tuberculosis included 12 men and 6 women at the age ranging from 19 to 56 years (mean, 29 years) with no evidence of HIV infection, neoplastic disease, or opportunistic infection. Of the 18 patients with tuberculosis, 9 (50%) had purely mesenteric tuberculous lymphadenopathy (TL) [presented with a small quantity of ascites (n = 3), thickened peritoneum (n = 2), thickened small bowel wall (n = 1), "dirty" great omentum (n = 1), renal tuberculosis (n = 1), hepato-duodenum ligament tuberculosis (n = 1), omental bursa tuberculosis (n = 1), pleuritis (n = 2)], and 9 (50%) had mesenteric TL accompanied with tuberculous mesenteritis (TLM) [presented with ascites (n = 8), thickened peritoneum (n = 8), thickened small bowel wall (n = 5), "dirty" great omentum (n = 6), "caked" great omentum (n = 3), infra-bowel abscess (n = 1), renal tuberculosis (n = 1), pleuritis (n = 1)]. All the patients with tuberculosis had constitutional symptoms, such as weight loss, easy fatigability, night sweats, and obscure abdominal pains. Five of them had clinically palpable abdominal masses. Tuberculosis was diagnosed if lymphadenopathy was found through pathologic examination of specimens (n = 8) taken at laparotomy or microbiologic examination of abdominal tissues (n = 3). Tuberculosis was also diagnosed in patients for whom CT showed improvement in documented tuberculosis at extra-abdominal sites after anti-tuberculous chemotherapy (n = 7).

No evidence of HIV infection was found in the 22 patients (including 10 men and 12 women at the age ranging from 20 to 74 years, mean 49 years) with newly diagnosed and previously untreated NH. The diagnosis was made by histologic examination of biopsy specimens of enlarged lymph nodes. Involvement of the left major psoas muscle in 2 patients and the adrenal gland in 1 patient was observed. Three patients had splenomegaly.

All patients giving their written informed consent were examined with a spiral CT scanner (Elscint HeliCAT Flash). Before undergoing CT, the patients drank 500 mL of a 1.5% diatrizoate solution. An 80-100 mL bolus of Ultravist (Schering Germany, 300 mgI/mL) at a rate of 2.5-3.0 mL/s was administered through veins. Contiguous axial images (5-8 mm thick) were obtained at 5-8 mm intervals from the dome of diaphragm to the symphysis pubis (120-140 KV, 212-250 Ma, pitch 1-1.5). Sixty seconds after injection of the contrast material, contrast-enhanced CT scan was performed.

Two observers unaware of the final diagnosis independently reviewed each CT image and recorded a number of characteristics of enlarged lymph nodes in SBM, including anatomic location, enhancement patterns and "sandwich sign". Discrepancies in interpretation between observers were solved by consensus. Small bowel mesenteric lymph nodes were grouped anatomically into the following three sites: the root, margin (area including mesenteric marginal vessels and vasa rectas) and body of SBM (the area between the root and margin of SBM). The short-axis diameter of each node was measured. The CT images of enlarged lymph nodes were compared with those of normal lymph nodes as previously described[12]. The enhancement patterns of enlarged lymph nodes in the 40 patients were described as homogeneous, peripheral, and homogeneously mixed peripheral enhancement. Enhancement was considered peripheral when thick, irregular or thin rim was seen, and homogeneously mixed peripheral when some enlarged nodes showed homogeneous enhancement and other nodes at the same site showed peripheral enhancement. Additionally, we observed the extranodal sites of tuberculosis and NHL, including spleen and abdominal wall. Differences in anatomic distribution, enhancement patterns and presence of "sandwich sign" between the two groups were compared by statistical analysis. Because of the small number of cases, Fisher's exact test was used to compare tuberculosis with NHL involving SBM.

RESULTS

The anatomic distribution and enhancement pattern findings are listed in Table 1 and the findings of "sandwich sign" are shown in Table 2.

CT revealed that TL and NHL affected mainly lymph nodes in the body and root of SBM (Figures 1 and 2). The margin of SBM was involved in NHL [5 patients (23%)], TL [1 patients (11%)], and TLM [0 patient (0%)].

Homogeneous enhancement (in the body of SBM) was found more often in NHL than in mesenteric TL and TLM ($P < 0.01$, $P < 0.05$). Homogeneously mixed peripheral enhancement (in the body of SBM) was observed more often in mesenteric TL and TLM than in NHL ($P < 0.05$). Homogeneous enhancement (in the root of SBM) was demonstrated more often in NHL than in TL ($P < 0.05$). Peripheral enhancement (in the root of SBM) was revealed more often in mesenteric TL and TLM than in NHL ($P < 0.01$). Enlarged lymph nodes

Table 2 Distribution of TL, TLM and NHL in mesenteric lymph nodes

	TL (n = 9)			TLM (n = 9)			NHL (n = 22)		
	Margin of SBM	Body of SBM	Root of SBM	Margin of SBM	Body of SBM	Root of SBM	Margin of SBM	Body of SBM	Root of SBM
Disperse	1	6	7	0	9	9	5	14	10
Confluence	0	2	2	0	0	0	0	8	12
Sandwich sign	0	1	1	0	0	0	0	6	12

TL: Tuberculous lymphadenopathy; TLM: Tuberculous lymphadenopathy accompanied with mesenteritis; NHL: Non-Hodgkin's lymphoma.

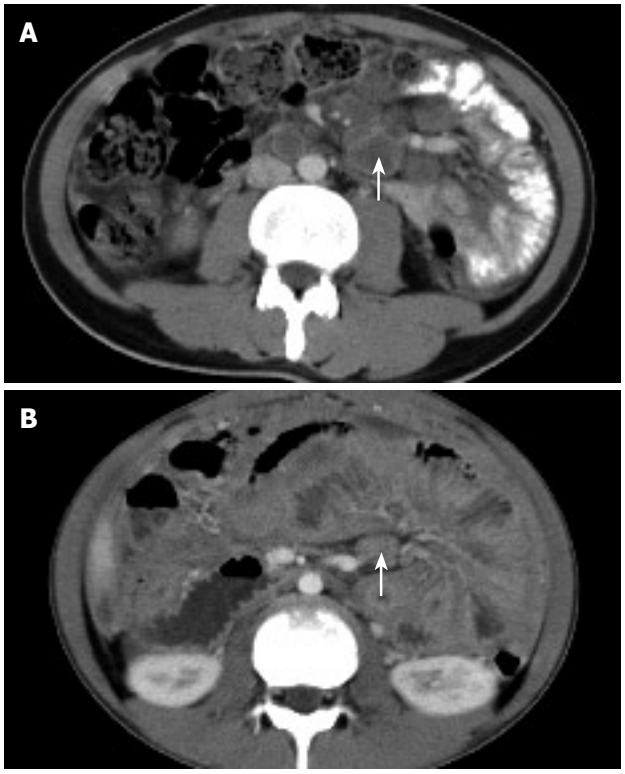

Figure 1 Contrast enhanced CT scan for a 25-year-old man with mesenteric TL showing enlarged lymph nodes in the body and root of SBM with peripheral enhancement (arrow) (**A**) and in the body of SBM with homogeneous enhancement (arrow) (**B**). The SBM was contracted and the wall of the small bowel was thickened.

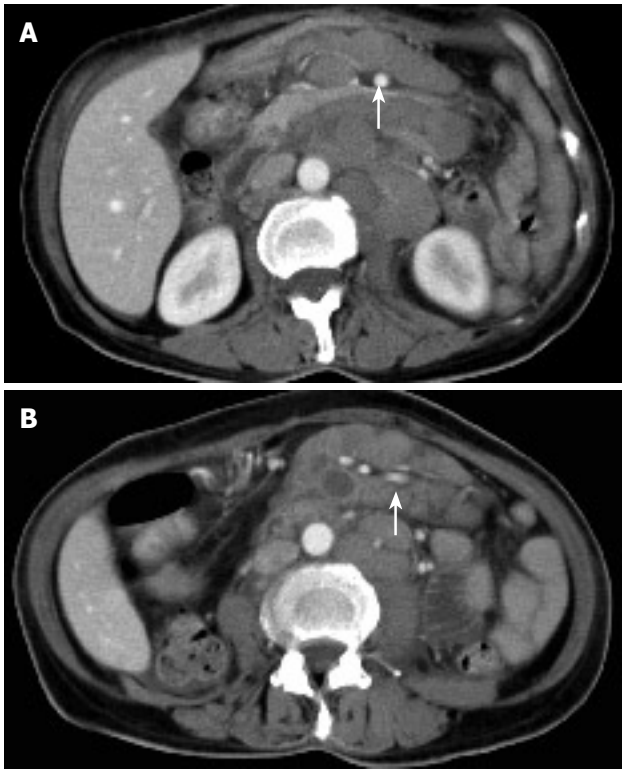

Figure 2 Contrast enhanced CT scan for a 56-year-old woman with NHL involving SBM showing enlarged lymph nodes in the root of SBM encasing the superior mesenteric artery (arrow), producing the "sandwich sign" (**A**) and homogeneously mixed peripheral enhancement of lymph nodes in the body of SBM encasing the small bowel mesenteric vessels (arrow), producing the "sandwich sign" (**B**).

(in the root of SBM) were dispersed in TLM, whereas confluence was found in NHL ($P < 0.01$). "Sandwich sign" (in the root of SBM) was displayed more often in NHL than in mesenteric TL and TLM ($P < 0.05$, $P < 0.01$).

DISCUSSION

TL is the most common manifestation of abdominal tuberculosis, and tuberculous infection may result in mesenteric lymphadenopathy[10,13]. It may be transmitted by three major routes. The first route is ingestion of materials infected with tubercle bacilli which are carried from a lesion in the intestinal submucosal layer to the lymph nodes draining the bowel segment. Drainage is usually from the lymphatics of the ileocecum, jejunum, ileum, and right side of colon to the peripancreatic and superior mesenteric lymph nodes. The second route is hematogenous spread. Bacteria are disseminated from a distant site of infection, usually the lungs, to the abdominal lymphatic system. Because this process is systemic, it may cause infection of mesenteric lymph nodes. The third route is infection spreading directly to the abdominal lymph nodes from the serosa of adjacent infected structures. Literature *et al*[14] reported that most patients with a past history of TB come from areas with a high prevalence of active tuberculosis and have epigastric pain, fever and weight loss, and enlarged nodules with focal calcification sometimes.

SBM, in a series of fan-like ruffles, suspends the jejunum and ileum to the posterior abdominal wall consisting of two posterior peritoneal layers. It is composed of fatty, extraperitoneal connective tissue, blood vessels, nerves, lymph nodes, and peritoneal

investment. The attached border of SBM root extends obliquely from the distal duodenum at the lower border of pancreas on the left side of L2 to the cecum in the right iliac fossa. The line of attachment passes from the duodenojejunal junction over the third portion of the duodenum, then obliquely across the aorta, inferior vena cava, right ureter and psoas major muscle, to the right ilica region[15-17].

The mesenteric lymph nodes can be divided into three subgroups: some lie close to the wall of small intestine, others occur in relation to primary branches of mesenteric vessels, and some consisting of central nodes along the main trunk of the superior mesenteric artery[18].

In this study, 18 patients with tuberculosis and 22 patients with NHL mainly involved mesenteric lymph nodes in the body and root of SBM. Hence, distribution of enlarged lymph nodes in the diseases closely paralleled to the anatomic distribution.

Pathologic findings from surgical specimens of TL indicated that caseation or liquefactive substances at the center of enlarged lymph nodes had a low attenuation presumably resulting from insufficient blood supply, whereas peripheral inflammatory lymphatic tissue had a higher attenuation on enhanced CT resulting from the preserved blood supply[19].

In most patients with untreated NHL, lymph nodes in the SBM increased homogeneously. In 9% of patients with NHL involving the body of SBM and 18% of patients with NHL involving the root of SBM, lymph nodes had a homogeneous and peripheral enhancement. Our findings on the morphology of lymph nodes are similar to those of previous reports, in which the enhancement patterns of untreated NHL are homogeneous or less frequently necrotic with central hypodensity in the neck and mediastinum[20-22].

In this study, 25% of patients had mesenteric TL in the body of SBM, lymph nodes had peripheral enhancement. In 50% of patients with mesenteric TL and 44% of patients with TLM in the root of SBM, lymph nodes had a homogeneous and peripheral enhancement, more than that in NHL.

The focus of this study was to differentiate mesenteric tuberculosis from untreated NHL involving the SBM using contrast-enhanced CT. Anatomic distribution in patients with NHL involving SBM was similar to that in patients with mesenteric TL and TLM. Oliver *et al*[23] reported that enlarged lymph nodes have a decreased density and mesenteric stranding in 20% of patients with lymphoma after treatment. However, if relapse of the disease occurs, the lymph nodes appear homogeneous, suggesting that it is important to know if patients with NHL have undergone therapy that may have caused central low attenuation within nodes or mesenteric stranding in SBM, simulating TL involving the SBM.

Neoplastic involvement of the mesentery can be diagnosed in lymphoma on the basis of a characteristic appearance of "sandwich-sign" encasement of the superior mesenteric artery[18,24]. In our study, one patient with TL had "sandwich-sign" in the body and root of SBM. However, it was detected in 27.3% of patients with NHL involving the body of SBM and in 54.5% of patients with NHL involving the root of SBM. When "sandwich-sign" is considered, mesenteric TL and TLM are rarely confused with NHL involving SBM in clinical practice.

One limitation of this study is the relatively small number of cases of tuberculosis involving SBM. Enlarged lymph nodes with a peripheral enhancement in SBM can also be seen in metastatic malignancy and other diseases. In general, if primary malignancy is known, most metastatic malignancies are easily diagnosed. Other causes for mesenteric lymphadenopathy that characteristically demonstrates central low attenuation on CT are Whipple disease[25] and cavitating mesenteric lymph node syndrome of celiac disease[26,27]. Mesenteric lymphadenopathy has also been reported in patients with familial Mediterranean fever during an acute abdominal attack, Castleman disease and Crohn's disease[28-31].

In conclusion, contrast-enhanced CT can be used in differentiating mesenteric TL and TLM from NHL involving SBM on the basis of enhancement patterns of enlarged lymph nodes and presence of "sandwich-sign". Mesenteric tuberculosis involves predominantly lymph nodes in the root and body of SBM. Lymph nodes at the margin of SBM are involved in only 5.5% of patients with mesenteric tuberculosis. In contrast, lymph nodes at the margin of SBM are involved in 22.7% of patients with NHL involving SBM. The presence of "sandwich sign" can be more frequently observed in NHL involving SBM than in mesenteric TL and TLM. A distinct difference in characteristic nodal enhancement patterns can also be observed.

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COMMENTS

Background

The incidence of tuberculosis is increasing. When the prevalence of abdominal tuberculosis is high, it is difficult to establish its diagnosis. Lymphadenopathy is the most common manifestation of abdominal tuberculosis and may be easily confused with lymphoma involving abdominal lymph nodes. Lymphoma is the most common malignant neoplasm affecting mesentery, and Hodgkin's Lymphoma rarely involves mesentery. We conducted a comparison of CT findings in tuberculosis and non-Hodgkin's lymphoma (NHL) involving small bowel mesentery (SBM) to improve physicians' ability to distinguish between these entities.

Research frontiers

The incidence of tuberculosis is increasing. Lymphadenopathy is the most common manifestation of abdominal tuberculosis. A comparison of CT findings in tuberculosis and lymphomas of retroperitoneal lymph nodes has been reported.

Innovations and breakthroughs

SBM, in a series of fan-like ruffles, suspends the jejunum and ileum to the posterior abdominal wall consisting of two posterior peritoneal layers. NHL and tuberculous lymphadenopathy may involve SBM, and the correct diagnosis and

differential diagnosis are important for their clinical treatment. We compared CT findings in tuberculosis and NHL involving SBM to improve the physicians' ability to distinguish between these entities.

Applications

This study may improve the physicians' ability to distinguish tuberculosis from NHL involving SBM, and specific CT imaging criteria may be used in the differential diagnosis of other malignant tumors involving SBM.

Peer review

This study evaluated the specific CT imaging criteria for differentiating tuberculosis involving small bowel mesenteric lymph nodes from lymphomas, showing that distribution of anatomic lymph nodes, sandwich sign and specific enhancement patterns of lymphadenopathy in SBM on CT images can be used in differentiating tuberculosis from NHL involving SBM. The study is well designed and interesting.

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CASE REPORT

A new approach to endoscopic treatment of tumors of the esophagogastric junction with individually designed self-expanding metal stents

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Abstract

The incidence of adenocarcinoma of the esophagogastric junction is constantly increasing. Curative treatment is no longer possible at the time of diagnosis in more than 50% of patients with esophageal carcinoma, and palliative treatment focusing on eliminating dysphagia is required. Endoscopic therapy with stent implantation is an established method of achieving this. It can be carried out quickly, with a low rate of early complications, and leads to fast symptomatic improvement, assessed using the dysphagia score. The relatively high rate of late complications such as stent migration, hemorrhage, and gastroesophageal mucosal prolapse has led to recent debate on the role of metal stents in palliative therapy. We present here a new type of stent design for transcardial application, which is intended to prevent bleeding due to mechanical mucosal lesions caused by the distal end of the stent extending into the stomach. The further intention of this case report is to force the discussion on individually designed nitinol stents in special anatomic conditions.

Key words: Esophagus cancer; Treatment; Palliative therapy; Endoscopic therapy; Stent; Cardiac cancer

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INTRODUCTION

The incidence of adenocarcinoma of the esophagogastric junction is constantly increasing[1,2]. Curative treatment is no longer possible at the time of diagnosis in more than 50% of patients with esophageal carcinoma, and palliative treatment[3] focusing on eliminating dysphagia is required. Endoscopic therapy with stent implantation is an established method of achieving this. It can be carried out quickly, with a low rate of early complications, and leads to fast symptomatic improvement, assessed using the dysphagia score[4-7]. The relatively high rate of late complications such as stent migration, hemorrhage, and gastroesophageal mucosal prolapse has led to recent debate on the role of metal stents in palliative therapy[8]. We present here a new type of stent design for transcardial application, which is intended to prevent bleeding due to mechanical mucosal lesions caused by the distal end of the stent extending into the stomach. The further intention of this case report is to force the discussion on individually designed nitinol stents in special anatomic conditions.

CASE REPORT

An 82-year-old patient presented at our department with 2-wk dysphagia, followed most recently by intermittent vomiting. He had a history of progressive prostate carcinoma (T3b Nx M0), which was treated with orchiectomy and antiandrogen therapy (bicalutamide). As there was local progression with infiltration of the urinary bladder, radiotherapy was planned. In addition, the patient had type 2 diabetes mellitus and arterial hypertension.

Gastroscopy showed a dilated esophagus, corresponding to the radiographic findings, with a high-grade stenosis 38 cm from the incisors, cranial to a 3-cm

Figure 1 Radiographic image of the esophagus stenosis.

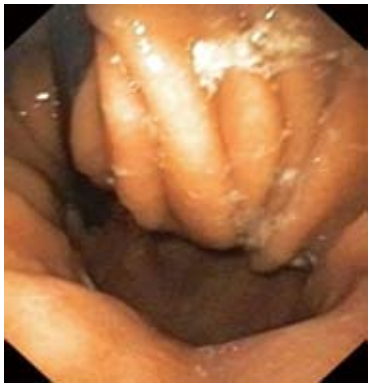

Figure 2 Endoscopic view of the hiatus hernia after passage through the tumor stenosis.

Figure 3 New stent design (Micro-Tech [Nanjing] Co. Ltd., Nanjing, China; distributed by Leufen Medizintechnik, Aachen, Germany).

long sliding hernia (Figures 1 and 2). The stenosis was diagnosed histologically as an adenocarcinoma of the esophagogastric junction, which already developed a hepatic metastasis. Reviewing the findings, we decided to carry out palliative stent implantation to treat the stenosis. As transcardially positioned stents are associated with higher complication rates[9] and experience shows that bleeding often occurs due to stent-related mucosal lesions in the stomach, we requested individual production of an unusually shaped self-expanding nitinol stent (SEMS) (Figure 3). Decisive factors in developing this new designed SEMS included the special anatomic conditions in this patient, with a medium-sized esophageal hernia. The stent was woven from nitinol in accordance with our specifications and was 140 mm long and 24 mm wide. A 30-mm wide bulb was formed at the cranial end of the stent to prevent stent migration. The distal end of the stent was to bend cranially to ensure that the stent would

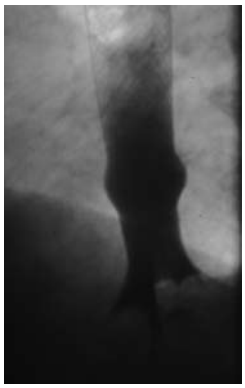

Figure 4 Radiographic image of the stent.

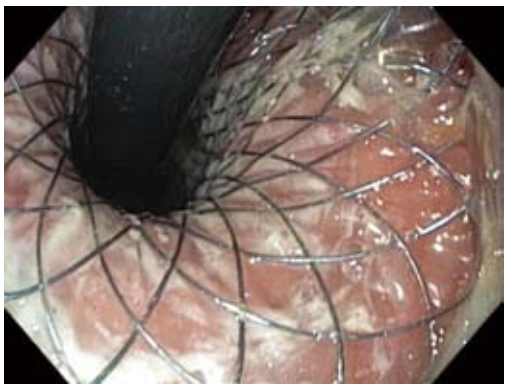

Figure 5 Endoscopic view of the distal end of the stent in inversion, 2 mo after treatment.

fit the cardia and not extend freely into the stomach. In addition, a circular ring-like widening was added 2 cm above the distal end of the stent to prevent migration. With the exception of the uncovered proximal bulb, the stent was completely covered.

After the patient's informed consent was obtained, the stent was placed on a carrier system with a diameter of 8 mm and released in the conventional way from the distal end by withdrawing an over tube. After the applicator with the stent was positioned fluoroscopically at the tumor level over a guide wire (Boston Scientific, Super Stiff Guidewire, 0.035 inch), the distal end of the stent was partially released. The proximally bent end of the stent was then pulled until the distal end fitted the cardia tightly. Finally, the stent was fully released by pulling and positioned without any special difficulties. The patient was able to take solid food 24 h later. A radiographic check showed that the stent was correctly positioned (Figure 4). No incidents of bleeding, vomiting as evidence of mucosal prolapse, or recurrent dysphagia occurred during a 4-mo follow-up period. An endoscopic check-up 2 mo after introduction of the stent showed that it was still correctly positioned, with no evidence of mucosal lesions (Figure 5).

DISCUSSION

Although the use of self-expanding metal stents is now an established part of palliative treatment for esophageal carcinomas, late complications are frequent.

The complication rate is higher when the stent is in a transcardial position. In our experience, however, stent migrations that are frequently reported occur much less often when a partially covered stent with a large diameter is used, and previous dilation of the tumor stenosis has not been carried out[10]. Nevertheless, bleeding, particularly due to mechanical lesions caused by the distal end of the stent which extends into the stomach, and mucosal prolapse with occlusion of the stent, still continue to be major problems. The aim in this case was therefore to take advantage of an individual stent design and provide an optimal solution in the palliative situation for this patient.

The stent described above was designed and manufactured in close collaboration with the producer and distributor of the nitinol stent within 10 d in order to avoid mechanical lesions of the mucosa in this special anatomic situation. The stent was positioned without any special difficulties in conventional way. The early clinical result was good after 24 h. The radiographic, clinical and endoscopic follow-up during a 4-mo period showed that the stent was still correctly positioned and none of usual complications occurred.

We believe that the stent design presented here is particularly suitable for palliative treatment of stenotic distal esophageal carcinomas. The advantage of this stent design is particularly clear when an axial hiatus hernia is present. Further optimization of the design and studies comparing it with conventional stents are required. We believe that individual stent designing could alleviate many of the late complications associated with stent treatment.

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CASE REPORT

Subcutaneous cervical emphysema and pneumomediastinum due to a lower gastrointestinal tract perforation

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Abstract

This case report describes a 69-year-old man presenting with an extensive subcutaneous emphysema in his neck and generalized peritonitis caused by a lower gastrointestinal tract perforation. This case emphasizes that subcutaneous emphysema patients with negative thoracic findings should be scrutinized for signs of retroperitoneal hollow viscus perforation.

Key words: Subcutaneous cervical emphysema; Pneumomediastinum; Gastrointestinal tract perforation; Malignancy; Diverticulitis

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INTRODUCTION

Pneumomediastinum usually occurs following esophageal or chest trauma. It can also occur spontaneously in association with asthma, excessive coughing, or straining. Cervical emphysema occurs when air moves through tissue planes into subcutaneous areas of the face and neck. Subcutaneous neck emphysema, pneumomediastinum, and retropneumoperitoneum have been reported infrequently following colonoscopic perforation. Iatrogenic colonic perforation is a serious but rare complication of colonoscopy. A perforation risk rate of 0.12% has been reported[1].

Subcutaneous emphysema caused by non-traumatic perforations of the colon is extremely rare. However, it should be considered when no obvious case can be found for the origin of subcutaneous emphysema or a pneumomediastinum.

CASE REPORT

A 69-year-old man presented himself at the Accident and Emergency Department with a 6-h history of swelling in the neck, an altered voice and abdominal pain. He was treated for a painful fifth rib on the left which was caused by a metastasis of an unknown primary tumor. Analysis for the primary tumor was ongoing, but not yet concluded. Radiotherapy was started and prednisolone was prescribed.

Physical examination revealed signs of extensive subcutaneous emphysema in his neck and generalized peritonitis. Laboratory blood and urine tests were normal apart from an elevated white cell count of 23×10^9 cells/L. A plain radiograph of the thorax showed free intraperitoneal air, pneumomediastinum and extensive subcutaneous emphysema, but no sign of pneumothorax (Figure 1).

An explorative laparotomy was performed, a perforation of the small bowel and the caecum was found, which were oversew. There were two perforations of the sigmoid but no palpable tumor. A sigmoid resection was performed with a permanent colostomy. The proximal end of the distal segment was oversew and left in place with a blind rectal pouch. The patient was admitted to the Intensive Care Unit after operation. Pathological examination showed diverticulitis with a perforation, and no tumor was found in the resected sigmoid.

After consultation with the patient and his family, the patient received no further surgical treatment. The patient died of respiratory arrest 12 d after surgery.

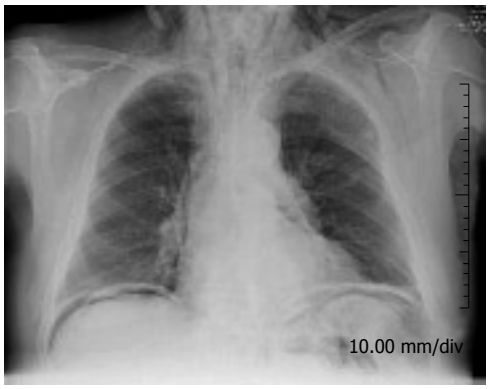

Figure 1 Thorax X-ray examination of the patient showing free air in abdomen, pneumomediastinum and severe subcutaneous cervical emphysema.

DISCUSSION

Non-traumatic subcutaneous emphysema is a rare presentation of lower gastrointestinal tract perforation due to colorectal cancer or diverticulitis[2-6].

As the rectosigmoid is located in the retroperitoneum, injury can be present in the absence of peritonitis. Mediastinal and cervical emphysema may develop due to dissection of air *via* contiguous tissue planes, which occurs along the perivascular adventitia to the anterior pararenal space, through the diaphragmatic hiatus along the adventitia of great vessels to the mediastinum and pericardium/or pretracheal fascia to the neck[7,8]. Subcutaneous emphysema patients with negative thoracic findings should be scrutinized for signs of retroperitoneal hollow viscus perforation to improve their outcome.

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CASE REPORT

Duplication cyst of the small intestine found by double-balloon endoscopy: A case report

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Abstract

A 35-year-old man was admitted due to bloody stool and anemia. The bleeding source could not be detected by esophagogastroduodenoscopy or colonoscopy. Double balloon endoscopy (DBE) revealed a diverticulum-like hole in which coagula stuck in the ileum at 1 meter on the oral side from the ileocecal valve. The adjacent mucosa just to the oral side of the hole was elevated like a submucosal tumor. The lesion was considered the source of bleeding and removed surgically. It was determined to be a cyst with an ileal structure on the mesenteric aspect accompanying gastric mucosa. The diagnosis was a duplication cyst of the ileum, which is a rare entity that can cause gastrointestinal bleeding. In the present case, DBE was used to find the hemorrhagic duplication cyst in the ileum.

Key words: Duplication cyst; Double-balloon endoscopy; Small intestine bleeding

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INTRODUCTION

Double-balloon endoscope (DBE) is a new endoscopic device designed to examine the small intestine. Observation of the entire small intestine can be achieved through a combination of anal and oral approaches. Endoscopic interventions such as mucosal biopsy, clipping, argon plasma and polypectomy can also be performed. We often encounter patients with obscure gastrointestinal bleeding in which bleeding cause cannot be revealed by the usual methods of esophagogastroduo denoscopy (EGD) and colonoscopy. DBE is thus used to find the origin of small intestinal bleeding. Here we report a case in which the origin of the patient's gastrointestinal bleeding was found with DBE and diagnosed as a duplication cyst of the ileum after surgery.

CASE REPORT

A 35-year-old man was admitted to a hospital with the complaint of right lower abdominal pain in April, 2003. Appendicitis was suspected and appendectomy was performed. However, he continued to suffer from occasional abdominal discomfort and symptoms of subileus. Early in August, bloody stools appeared. Iron deficiency anemia (hemoglobin level, 8.1 g/dL) was also noted. Although EGD, colonoscopy and

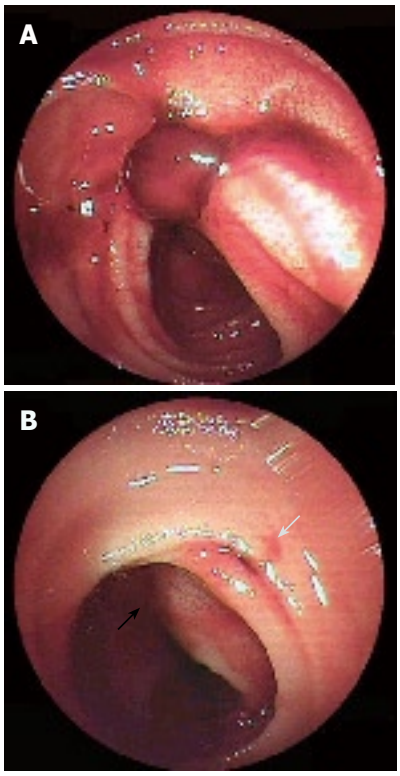

Figure 1 Double-balloon endoscopy by a perianal approach revealing coagula in the ileum at 1 m on the oral side of the ileocecal valve (A) and a diverticulum-like hole (white arrow) after removal of the coagula with an elevation like a submucosal tumor on the oral side of the hole (black arrow) (B).

radiological enteroclysis were performed, the origin of the gastrointestinal bleeding remained unknown.

At the end of August 2003, the right lower abdomen pain appeared again after a meal. Inflammatory reactions were also elevated. Thus, he was referred to our hospital for further examination. Physical examination showed local peritonitis. Abdominal CT displayed ascites in the circumference of the liver and thickness of the distal small intestine wall in the pelvis. With appropriate medical treatment and antibiotic therapy, his condition and the inflammatory reaction improved. Colonoscopy and radiological enteroclysis failed to ascertain any lesions responsible for the symptoms. The patient was thus discharged from hospital.

His condition was good until bloody stool suddenly appeared again in July, 2005. He was admitted to our hospital the next day. Anemia gradually progressed. Scintigraphy for hemorrhage revealed a deposit in the ileum end. Colonoscopy, however, failed to detect the source of the bleeding. The patient was then referred to Kyushu University Hospital. DBE performed using a perianal approach revealed coagula adhered to the ileal wall about 1 m on the oral side from the ileocecal valve (Figure 1A). When the coagula were removed, a bleeding diverticulum-like hole appeared and the adjacent mucosa just on the oral side of the hole was elevated like a submucosal tumor (Figure 1B). We injected hypertonic saline with epinephrine and also injected India ink near it as a marker. However, intermittent bleeding continued after the procedure. The patient, therefore, underwent

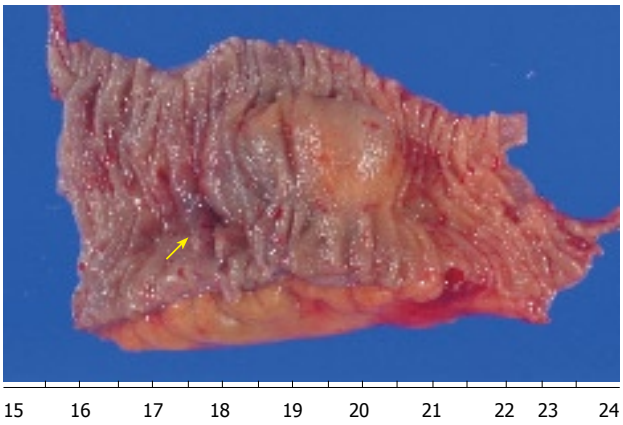

Figure 2 Macroscopic appearance of the resected specimen. The cyst was located on the mesenteric aspect and a diverticulum-like hole (arrow) was detected on the anal side.

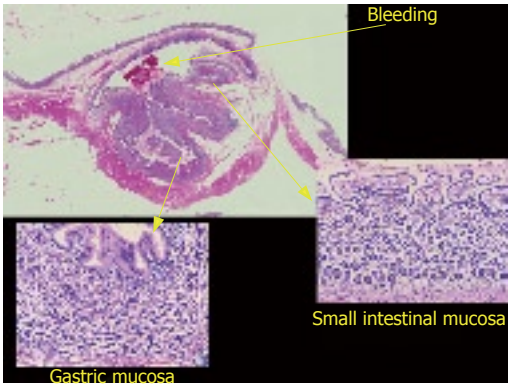

Figure 3 HE staining for the resected tissue showing an ileal structure with gastric mucosa and bleeding in the cyst.

an urgent operation. When the inside of the abdominal cavity was observed, a cyst was found on the mesenteric side of the ileum, about 1mm proximal to the ileocecal valve, and connected to the ileocecal valve by an adhesion band. The lesion was considered the bleeding source and thus resected. Histological examination revealed that the cyst was located on the mesenteric aspect and had an ileal structure with gastric mucosa (Figures 2 and 3). A diagnosis of duplication cyst of the ileum was thus made. The patient was discharged from hospital 10 d after surgery and has remained symptom-free since then.

DISCUSSION

Duplication cysts are congenital malformations that can arise throughout the alimentary tract from the oral cavity to the anus. The majority are diagnosed in infancy and childhood. Duplication cysts of the small intestine constitute about 60% of those in alimentary tract and are located on the mesenteric aspect, in contrast to a Mekel's diverticulum that localizes on the antimesenteric aspect. Duplication cysts are classified into spherical and tubular types. The former is more commonly found in the small intestine[1]. The present case was considered a

spherical one.

Hoshi *et al*[2] reported that 34% of duplication cyst patients complain of stomachache, 24% vomiting, 17% an abdomen mass and 10.5% bloody stool. In our case, because of the right lower abdominal pain, appendectomy was performed 2 years ago. After the operation, the various symptoms continued. Though these symptoms seemed to be due to a duplication cyst, the relevant diagnosis was not made until 2 years later.

A preoperative diagnosis of duplication cyst is difficult. Indeed, only 11.2% have been correctly diagnosed before operation in Japan. Eighteen point two percent are diagnosed as intussusception, 15.1% as an abdominal mass, 14.4% as ileus, and 26.7% are not able to be diagnosed[2]. Cases diagnosed before operation have large lesions that are detectable by abdominal CT and US[3].

Previously, it was relatively difficult to diagnose a small intestinal lesion. However, diagnostic strategy changes with the availability of DBE[4] and wireless capsule endoscopy (CE). In fact, Toth *et al*[5] reported that they could find a tubular type duplication cyst of the small intestine by CE, suggesting that CE can reveal circumferentially ulcerated stenosis in the ileum. There is no report on an image of a spherical type of duplication cyst in the small intestine detected by endoscopy. Here, for the first time, we showed an image of a spherical type of duplication cyst obtained by DBE. The cyst was too small to be detected by other modalities such as CT, US and radiological enteroclysis.

Duplication cysts are rare in adults and there are few reports on a diagnosis made before operation. Thus ascertaining the existence of such a lesion is difficult. We presented a case of a duplication cyst of the small intestine found by DBE. We expect that duplication cysts may be more frequently found with the future spread of DBE and CE.

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CASE REPORT

Intraperitoneal metastasis of hepatocellular carcinoma after spontaneous rupture: A case report

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Abstract

Rupture of hepatocellular carcinoma (HCC) is a life-threatening complication. Peritoneal metastasis of HCC after spontaneous rupture was seldom noted. We report a case of intraperitoneal metastasis of HCC after spontaneous rupture. A previously asymptomatic 72-year-old man was admitted due to dull abdominal pain with abdominal fullness. He had a history of HCC rupture 10 mo ago and transarterial embolization was performed at that time. Abdominal computer tomography (CT) scan showed a huge peritoneal mass over the right upper quadrant area. Surgical resection was arranged and subsequent microscopic examination confirmed a diagnosis of moderately-differentiated HCC.

Key words: Hepatocellular carcinoma; Spontaneous rupture; Peritoneal metastasis

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide. It is usually manifested in the 6th and 7th decades of life. Extrahepatic metastases are seen in 64% of patients with HCC. The most frequent sites of extrahepatic metastases are lung, abdominal lymph node and bone, but peritoneal dissemination is unusual[1,2]. The incidence of spontaneous rupture of HCC is about 8%-26% in Asia[3-5] and the mortality rate of HCC patients is 10%[6]. However, peritoneal metastasis of HCC after spontaneous rupture is seldom noted. Here, we report a case of intraperitoneal metastasis of HCC after spontaneous rupture 10 mo ago, which was treated with transarterial embolization.

CASE REPORT

A previously asymptomatic 72-year-old man had a history of chronic hepatitis C- related liver cirrhosis without regular follow-up. Sudden nausea and vomiting with watery diarrhea were noted on January 2006. Then he was sent to Yun-Lin Branch of National Taiwan University Hospital for help. Abdominal computer tomography (CT) scan showed a huge HCC that was suspicious of rupture. Under the request of his family, he was transferred to our hospital and transarterial embolization was performed on January 31, 2006. After discharge, he was regularly followed up at our Gastrointestinal (GI) Outpatient Department (OPD). Dull abdominal pain over the right upper quadrant area, accompanied with fullness sensation, was noted in November 2006. Besides, he also had body weight loss of about ten kilograms in one year. So he visited our hospital again. Abdominal CT scan revealed a peritoneal mass in the right upper quadrant peritoneal area and hepatoma recurrence was considered (Figure 1). Transarterial embolization was arranged again, but failed. After consultation with the surgeon, he was admitted for surgical resection.

Surgical intervention was arranged on January 24, 2007. Operative methods were segmental hepatectomy (S6 and partial S5), excision of extrahepatic tumor, and cholecystectomy. The operation showed a huge tumor (12 cm × 8 cm × 6 cm) over the right upper quadrant area just below liver parenchyma (Figure 2) with its blood supplied from the omentum. Besides, two small mass lesions (3 cm × 2 cm and 2 cm × 1 cm) were found over

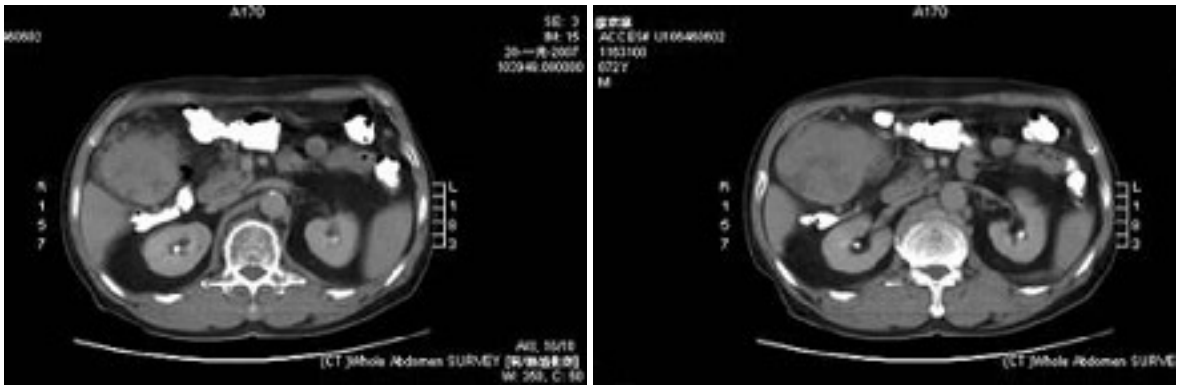

Figure 1 A well-defined mass about 10 cm in diameter in RUQ peritoneal cavity anterior to liver parenchyma.

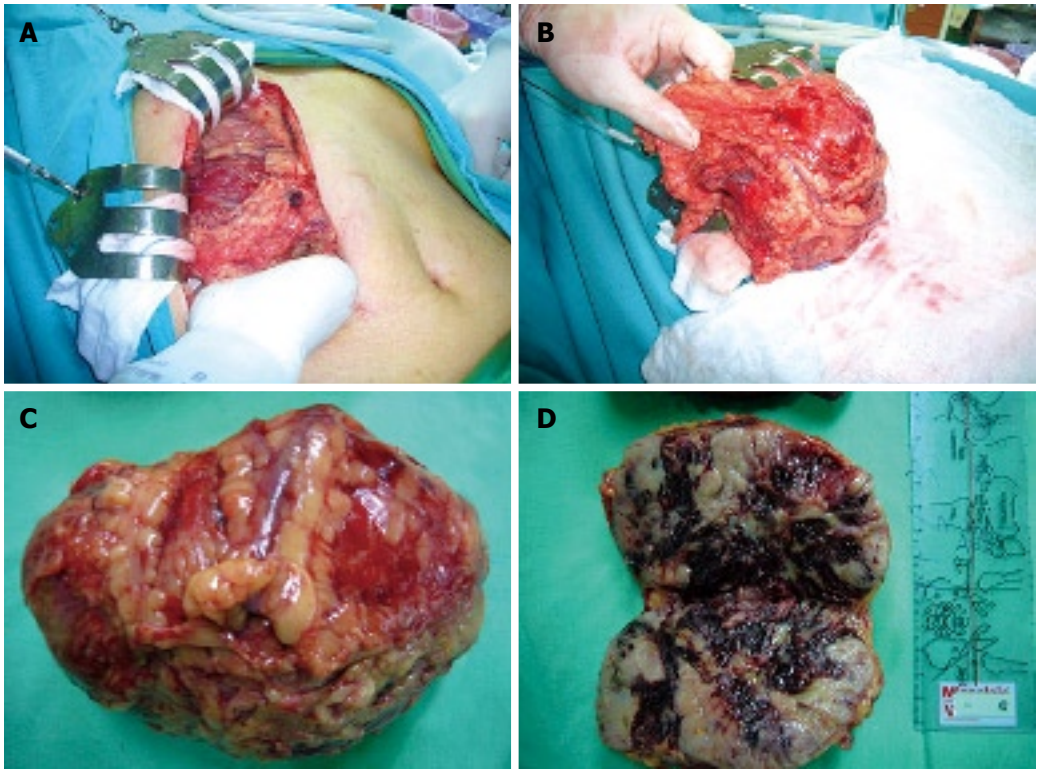

Figure 2 A huge tumor (12 cm × 8 cm × 6 cm) over the right upper quadrant area just below liver (**A**), blood supply of tumor from the omentum (**B**), and intraperitoneal tumor (**C**, **D**).

S5 and S6, respectively (Figure 3). Microscopy showed that the huge extrahepatic tumor and two intrahepatic lesions were moderately differentiated.

DISCUSSION

Intraperitoneal metastasis of non-ruptured HCC is rare, but the risk of such a metastasis of ruptured HCC increases[7]. The mechanism of spontaneous rupture of HCC is not exactly clear. Hypotheses include rapid expansion of the tumor and central necrosis, venous hypertension caused by venous obstruction due to direct tumor invasion, mild trauma or compression by the diaphragm associated with respiratory movement, coagulopathies such as thrombocytopenia and disturbed prothrombin synthesis, and vascular injury leading to hemorrhage and subsequent rupture[4,8-10]. Recently, Zhu *et al* postulated that the poor function of macrophage phagocytosis could result in cumulation of immune complex and deposition on vascular wall. Then vascular wall could become stiff and weak due to the proliferated fragment elastin and damaged collagen, which would make blood vessels more prone to splitting and result in hemorrhage and rupture of HCC[11,12]. Large tumor size, peripheral location and protruding contour are all associated with an increased risk for rupture of HCC[4,13,14].

The diagnosis of HCC rupture is based on blood-stained ascites plus imaging studies and symptoms[2]. The most common symptom is sudden onset of abdominal pain (66%-100%)[6,15-17]. Yeh *et al* also found that the presence of sudden-onset abdominal pain is the only independent indicator of ruptured HCC[18]. Abdominal ultrasonography and computed tomography improve the rate of preoperative diagnosis[13,14,19,20]. Peripheral location, protruding contour, discontinuity of hepatic surface, surrounding hematoma and elevated ascitic CT number are helpful signs for the diagnosis of ruptured HCC. In addition, enucleation sign on helical CT could

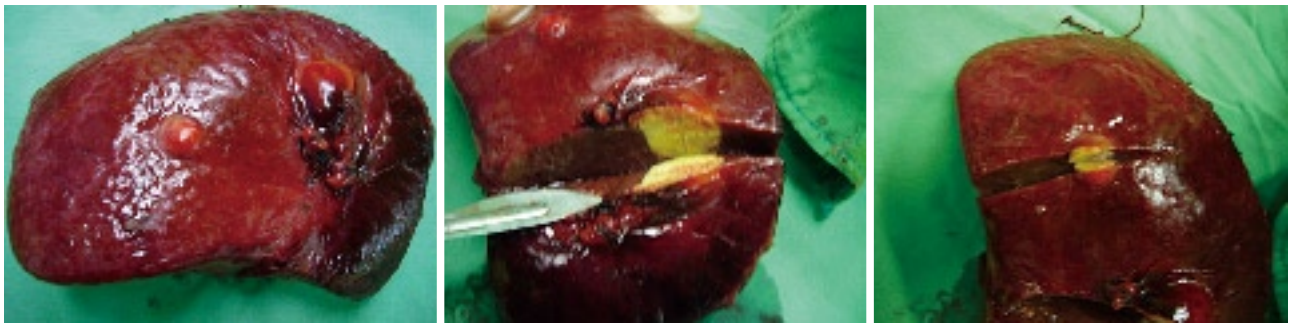

Figure 3 Two small mass lesions (3 cm × 2 cm and 2 cm × 1 cm) over S5 and S6, respectively.

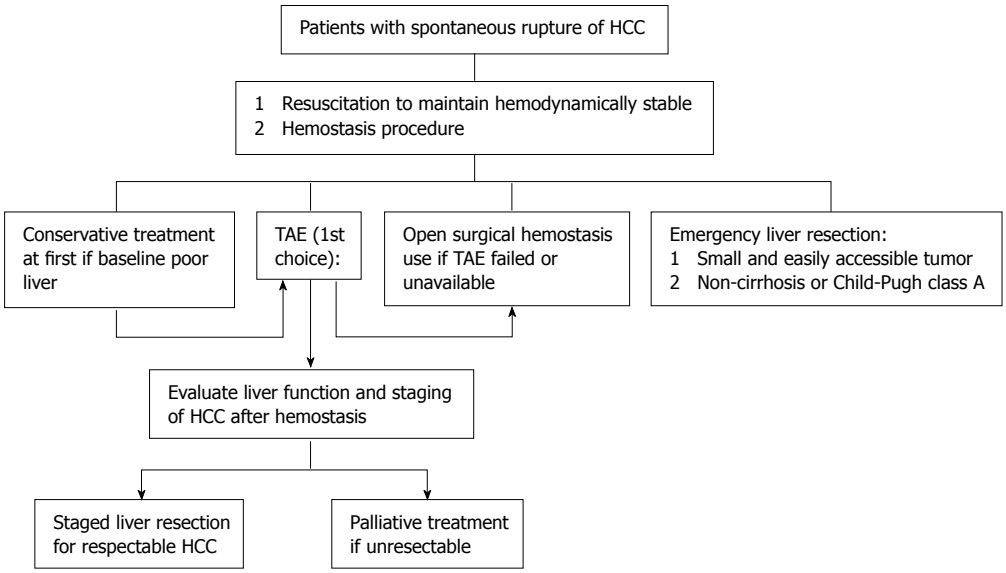

Figure 4 Logarithm about how to approach to the patient with spontaneously ruptured HCC.

be more specific[14,20,21].

Treatment of ruptured HCC is primarily aimed at controlling hemorrhage and preserving the functional liver parenchyma as possible. Open surgical method was the mainstay of treatment during 1960s-1980s. It was reported that various surgical procedures, including perihepatic packing, suture plication of bleeding tumors, injection of alcohol, hepatic artery ligation (HAL), and liver resection, are effective against hemostasis[10,22-26]. Besides transarterial embolization (TAE) and transarterial chemoembolization (TACE) for palliative treatment in patients with unresectable HCC, TAE is also gradually used for hemostasis in spontaneous rupture of HCC. In addition, Ng *et al*[27] also reported that ruptured HCC could be treated with radiofrequency ablation as a salvage procedure. To our knowledge, no prospective randomized controlled trials have found the best method for hemostasis. There is evidence that TACE is the preferred method to arrest tumor bleeding[13,28-34]. Besides, two-stage hepatectomy is advisable because it can prolong the survival of selected patients[35-40]. Figure 4 is the logarithm about how to approach the patients with spontaneous ruptured HCC[35-40].

Spontaneous rupture of HCC with intraperitoneal hemorrhage is a life-threatening complication with a high mortality rate. Prognosis is associated with poor liver reserve, advanced disease and severity of hemorrhage[41]. The median survival time is around 4-5 mo after HCC rupture, and only few patients can have a long-term survival[42,43]. Thus, implanted metastases usually do not become clinically apparent. Ong *et al*[44] reported the first case of peritoneal metastasis after HCC rupture in 1996 and then only sporadic case reports have been published. Most reported cases of peritoneal metastases are documented 8 mo after rupture[7,41,45-47], but Ryu *et al*[48] and Lin *et al*[49] reported that peritoneal metastasis can be found 4 and 3 mo respectively after rupture episode. A single metastatic tumor is the most common presentation. Resection is the treatment of choice for peritoneal metastasis if possible and might offer long-term survival benefits[7,44-48].

Peritoneal metastasis after spontaneous rupture of HCC is rare. This is our first experience with such a patient. Our patient developed peritoneal metastasis, which was documented 10 mo after spontaneous HCC rupture. The time from rupture to documentation of peritoneal metastasis is similar to other case reports. Because few cases of peritoneal metastasis after ruptured HCC have been reported, the association between metastatic tumor and viral infection (HBV or HCV), AFP level, or age is lacking. Lin *et al*[49] found that most reported cases are males, but the impact of gender is still unclear. The disease-free time is around 7-45 mo according to previous case reports[40-42,44]. Our patient had no peritoneal

recurrence until December, 2007 and is now regularly followed up at OPD.

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Meetings

Events Calendar 2008-2009

FALK SYMPOSIA 2008
January 24-25, Frankfurt, Germany
Falk Workshop: Perspectives in Liver
Transplantation

International Gastroenterological Congresses 2008
February 14-16, Paris, France
EASL-AASLD-APASL-ALEH-IASL Conference Hepatitis B and C virus resistance to antiviral therapies
www.easl.ch/hepatitis-conference

February 14-17, Berlin, Germany
8th International Conference on New Trends in Immunosuppression and Immunotherapy
www.kenes.com/immuno

February 28, Lyon, France
3rd Congress of ECCO - the European Crohn's and Colitis Organisation
Inflammatory Bowel Diseases 2008
www.ecco-ibd.eu

February 29, Québec, Canada
Canadian Association of Gastroenterology
E-mail: general@cag-acg.org

March 10-13, Birmingham, UK
British Society of Gastroenterology Annual Meeting
E-mail: BSG@mailbox.ulcc.ac.uk

March 14-15, HangZhou, China
Falk Symposium 163: Chronic Inflammation of Liver and Gut

March 23-26, Seoul, Korea
Asian Pacific Association for the Study of the Liver
18th Conference of APASL: New Horizons in Hepatology
www.apaslseoul2008.org

March 29-April 1, Shanghai, China
Shanghai-Hong Kong International Liver Congress
www.livercongress.org

April 05-09, Monte-Carlo (Grimaldi Forum), Monaco
OESO 9th World Congress, The Gastro-esophageal Reflux Disease: from Reflux to Mucosal Inflammation-Management of Adeno-carcinomas
E-mail: robert.giuli@oeso.org

April 9-12, Los Angeles, USA
SAGES 2008 Annual Meeting - part of Surgical Spring Week
www.sages.org/08program/html/

April 18-22, Buenos Aires, Argentina
9th World Congress of the International Hepato-Pancreato Biliary Association
Association for the Study of the Liver
www.ca-ihpba.com.ar

April 23-27, Milan, Italy
43rd Annual Meeting of the European Association for the Study of the Liver
www.easl.ch

May 2-3, Budapest, Hungary
Falk Symposium 164: Intestinal Disorders

May 18-21, San Diego, California, USA
Digestive Disease Week 2008

May 21-22, California, USA
ASGE Annual Postgraduate Course
Endoscopic Practice 2008: At the Interface of Evidence and Expert Opinion
E-mail: education@asge.org

June 4-7, Helsinki, Finland
The 39th Nordic Meeting of Gastroenterology
www.congrex.com/ngc2008

June 5-8, Sitges (Barcelona), Spain
Semana de las Enfermedades Digestivas
E-mail: sepd@sepd.es

June 6-8, Prague, Czech Republic
3rd Annual European Meeting: Perspectives in Inflammatory Bowel Diseases
E-mail: meetings@imedex.com

June 10-13, Istanbul, Turkey
ESGAR 2008 19th Annual Meeting and Postgraduate Course
E-mail: fca@netvisao.pt

June 11-13, Stockholm, Sweden
16th International Congress of the European Association for Endoscopic Surgery
E-mail: info@eaes-eur.org

June 13-14, Amsterdam, Netherlands
Falk Symposium 165: XX International Bile Acid Meeting. B ile Acid Biology and Therapeutic Actions

June 13-14, Prague, Czech Republic
Central and Eastern European Conference on Colorectal "Cancer" Screening, Prevention and Management
E-mail: idca2008@guarant.cz

June 25-28, Barcelona, Spain
10th World Congress on Gastrointestinal Cancer
Imedex and ESMO
E-mail: meetings@imedex.com

June 25-28, Lodz, Poland
Joint Meeting of the European Pancreatic Club (EPC)
and the International Association of Pancreatology (IAP)
E-mail: office@epc-iap2008.org
www.e-p-c.org
www.pancreatology.org

June 26-28, Bratislava, Slovakia
5th Central European Gastroenterology Meeting
www.ceurgem2008.cz

July 9-12, Paris, France
ILTS 14th Annual International Congress
www.ilts.org

September 10-13, Budapest, Hungary
11th World Congress of the International Society for Diseases of the Esophagus
E-mail: isde@isde.net

September 13-16, New Delhi, India
Asia Pacific Digestive Week
E-mail: apdw@apdw2008.net

III FALK GASTRO-CONFERENCE
September 17, Mainz, Germany
Falk Workshop: Strategies of Cancer Prevention in Gastroenterology

September 18-19, Mainz, Germany
Falk Symposium 166:
GI Endoscopy - Standards & Innovations

September 18-20, Prague, Czech Republic
Prague Hepatology Meeting 2008
www.czech-hepatology.cz/phm2008

September 20-21, Mainz, Germany
Falk Symposium 167:
Liver Under Constant Attack - From Fat to Viruses

September 24-27, Nantes, France
Third Annual Meeting
European Society of Coloproctology
www.escp.eu.com

October 8-11, Istanbul, Turkey
18th World Congress of the International Association of Surgeons,
Gastroenterologists and Oncologists
E-mail: orkun.sahin@serenas.com.tr

October 18-22, Vienna, Austria
16th United European Gastroenterology Week
www.negf.org
www.acv.at

October 22-25, Minnesota, USA
Anstralian Gastroenterology Week 2008
E-mail: gesa@gesa.org.au

October 22-25, Brisbane, Australia
71st Annual Colon and Rectal Surgery Conference
E-mail: info@colonrectalcourse.org

October 31-November 4, Moscone West Convention Center, San Francisco, CA
59th AASLD Annual Meeting and Postgraduate Course
The Liver Meeting
Information: www.aasld.org

November 6-9, Lucerne, Switzerland
Neurogastroenterology & Motility Joint International Meeting 2008
E-mail: ngm2008@mci-group.com
www.ngm2008.com

November 12, Santiago de Chile, Chile
Falk Workshop: Digestive Diseases: State of the Art and Daily Practice

November 28-29, Cairo, Egypt
1st Hepatology and Gastroenterology Post Graduate Course
www.egyptgastrohep.com

December 7-9, Seoul, Korea
6th International Meeting
Hepatocellular Carcinoma: Eastern and Western Experiences
E-mail: sglee@amc.seoul.kr

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FALK FOUNDATION e.V.
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Advanced Courses - European Institute of Telesurgery EITS - 2008
Strasbourg, France
January 18-19, March 28-29, June 6-7, October 3-4

N.O.T.E.S
April 3-5, November 27-29
Laparoscopic Digestive Surgery

June 27-28, November 7-8
Laparoscopic Colorectal Surgery

July 3-5
Interventional GI Endoscopy Techniques
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March 23-26, Glasgow, Scotland
Meeting of the British Society of Gastroenterology (BSG)
E-mail: bsg@mailbox.ulcc.ac.uk

May 17-20, Denver, Colorado, USA
Digestive Disease Week 2009

November 21-25, London, UK
Gastro 2009 UEGW/World Congress of Gastroenterology
www.gastro2009.org

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