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TOPIC HIGHLIGHT

Paolo Gionchetti, MD, *Series Editor*

Imaging of the small bowel in Crohn's disease: A review of old and new techniques

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Abstract

The investigation of small bowel morphology is often mandatory in many patients with Crohn's disease. Traditional radiological techniques (small bowel enteroclysis and small bowel follow-through) have long been the only suitable methods for this purpose. In recent years, several alternative imaging techniques have been proposed. To review the most recent advances in imaging studies of the small bowel, with particular reference to their possible application in Crohn's disease, we conducted a complete review of the most important studies in which traditional and newer imaging methods were performed and compared in patients with Crohn's disease. Several radiological and endoscopic techniques are now available for the study of the small bowel; each of them is characterized by a distinct profile of favourable and unfavourable features. In some cases, they may also be used as complementary rather than alternative techniques. In everyday practice, the choice of the technique to be used stands upon its availability and a careful evaluation of diagnostic accuracy, clinical usefulness, safety and cost. The recent development of innovative imaging techniques has opened a new and exciting area in the exploration of the small bowel in Crohn's disease patients.

Key words: Crohn's disease; Small bowel; Imaging techniques; Ultrasonography; Magnetic resonance; Computed tomography; Video-capsule endoscopy; Double-balloon endoscopy

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INTRODUCTION

The small bowel has been defined for many years as the "black box" of the gastrointestinal system due to its inaccessibility to endoscopic exploration. Indeed, until recently, the only available endoscopic technique to explore the small bowel, the so-called push enteroscopy, was very seldom able of a complete small bowel exploration; this may become an important limit, particularly in Crohn's disease which often involves the most distal portion of the ileum[1].

Therefore, the conventional radiological methods, i.e. small bowel enteroclysis (SBE) and small bowel follow-through (SBFT), have long been the only imaging methods providing information on the morphological features of the small bowel valuable in the diagnosis and management of Crohn's disease.

In the last few years, several alternative techniques have been proposed for imaging of the small bowel. Indeed, the evaluation of bowel wall features, as well as of extra-intestinal involvement by bowel ultrasound (US), has gained diffuse popularity. At the same time, developments in hardware and software components, as well as in the use of contrast media, have led Magnetic Resonance (MR) and Computed Tomography (CT) to have a recognized role in the study of intestinal diseases and of their intra-abdominal complications.

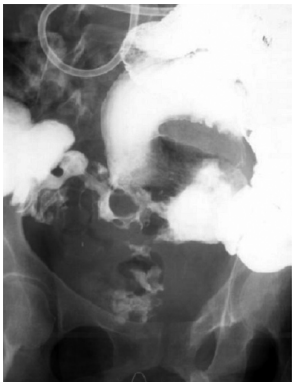

Figure 1 Small bowel enteroclysis. Evidence of narrowing and alterations in the terminal ileum and presence of an entero-vesical fistula.

Furthermore, recently the direct visualization of the mucosa of the whole small bowel has become possible thanks to the development of video-capsule endoscopy (VCE) and of "double-balloon" enteroscopy.

The aim of this review is to point out the most recent advances and the advantages and limitations of the various imaging techniques now available for the study of the small bowel, with particular reference to their use in patients with suspected or proven Crohn's disease.

CONVENTIONAL RADIOLOGY

As stated before, investigation of the small bowel in Crohn's disease has been based for a long time on radiological techniques, such as small bowel enteroclysis (SBE) and small bowel follow-through (SBFT).

Typical small bowel changes which can be observed by means of these techniques include irregular thickening and distortion of the valvulae conniventes, loops adhesions (mass-like effect) or separated loops because of wall thickening and mesenteric inflammatory infiltration. Transverse and longitudinal distribution of ulcerations can separate islands of thickened internal wall, resulting in the typical cobblestone appearance. Strictures are often separated by healthy bowel tracts (skip lesions); impaired small bowel peristalsis is commonly observed within rigid stenotic tracts. Extrinsic compression may be observed, due to mesentery lymph node enlargement[2,3].

The conventional technique was historically able to differentiate between Crohn's disease, granulomatous enteritis and ulcerative colitis[2,3].

Both SBE and SBFT, when performed by experienced examiners, appear to be characterized by similar sensitivity (85%-95%) and specificity (89%-94%) in detecting the radiological lesions typical of Crohn's disease. The preference for one technique or the other largely depends on institutional standards[4,5] while the preference of patients is usually in favour of SBFT since no nasal (or oral) intubation is required. SBFT is also usually associated with a lower radiation exposure and is a less expensive and time consuming examination; moreover, SBFT does not miss duodenal disease[6].

Both procedures are able to evaluate small bowel peristalsis, the intra-abdominal distribution of bowel loops, the presence of strictures and dilatations, the distensibility of the intestinal lumen, the presence of fistulae (Figure 1), the morphology of circular folds and other features of the mucosal surface. All this information is very valuable in the evaluation of patients with Crohn's disease.

Also, since SBE and SBFT have represented the standard approach for a long time, they are now commonly used as terms of comparison in evaluating the diagnostic accuracy of all new imaging techniques.

Table 1 Main US features observed in Crohn's disease (modified from Maconi *et al*[93])

Features	Crohn's disease
Bowel wall	
Thickening	4-14 mm
Echopattern	Variable
Vascularity	Variable
Contours	Variable
Stiffness	Often present
Haustra coli	Absent
Peristalsis	Often weak or absent
Location and extension	
Site	Ileum 70%
Bowel involvement	Often divided into segments
Extra-intestinal alterations	
Mesenteric hypertrophy	Common
Enlarged regional lymph nodes	Common
Fistulae and abscesses	Common

BOWEL ULTRASOUND

The first attempts to study Crohn's disease by means of ultrasound (US) date back to the late Seventies[7]. Since then, many studies have addressed the possible role of this technique in diagnosing and monitoring Crohn's disease with continuous improving quality in US imaging due to evolving technological advancements.

The main ultrasound findings in Crohn's disease are represented by thickening and stiffness of the gut wall, modifications or lack of its echostratification, reduction of peristalsis, mesenteric fibro-fatty proliferation, lymph node enlargement; in case of complications, narrowing of the intestinal lumen, abscesses and fistula are usually easily detectable (Table 1).

At least four studies have prospectively compared the diagnostic accuracy of US with that of radiological studies, endoscopy or surgery in suspected Crohn's disease at diagnosis[8-10]. In these studies, the sensitivity of US ranged between 84% and 90% and its specificity reached 98%-100%.

The capacity of US to detect the exact location of Crohn's disease was also compared with the findings at radiology, endoscopy or surgery in some studies[11-16]. In the largest series[12], the overall sensitivity was 93% and the specificity was 97%, the highest sensitivity (95%) being reached when the disease involved the terminal ileum.

The use of US has also been proposed in the follow-up of patients with known Crohn's disease, not only when an abdominal complication (i.e. strictures, abscesses or fistula) is suspected, but also in asymptomatic patients in order to identify the occurrence of complication(s) at an earlier stage[17].

Indeed, US compares very well with radiological techniques and surgery, mainly in the detection of

Table 2 Detection of strictures in Crohn's disease: comparison between US and conventional radiology or surgery (prospective studies)

Author (ref)	Patients (*n*)	Comparator	Sensitivity	Specificity
Maconi *et al*[18]	98	Small bowel/ barium enema	74%	93%
Kohn *et al*[20]	44	Small bowel enema	82%	100%
Gasche *et al*[19]	33	Surgery/pathology	100%	91%
Parente *et al*[12]	211	Small bowel enema	79%	98%
	85 (operated)	Surgery	90%	100%

Adapted from Parente *et al*[22].

Table 3 Detection of intra-abdominal abscesses in Crohn's disease: comparison between US and CT or surgery (prospective studies)

Author (ref)	Patients (*n*)	Comparator	Sensitivity	Specificity
Maconi *et al*[18]	58	CT scan	83%	94%
Gasche *et al*[19]	33	Surgery/pathology	100%	92%
Maconi *et al*[21]	128	Surgery	91%	87%

Adapted from Parente *et al*[22].

strictures and abscesses, while for fistulas data are more conflicting and less satisfactory, with sensitivity values ranging from 50% to 87% and specificity values from 90% to 95%[12,18-22] (Tables 2 and 3).

Repeated US examinations may be of help in the follow-up of Crohn's disease patients after surgery; indeed, in this setting US is able to detect endoscopic recurrence after resective surgery[23,24]. Also, in one study, in non operated patients, increased bowel thickness was the major risk factor for intestinal resection in the following 12 mo[25]; its persistence after conservative surgery appears to identify those patients at highest risk of clinical and surgical recurrence[26].

The use of US has also been proposed as a possible tool in the assessment of disease activity. However, a weak correlation between US findings and clinical disease severity is usually observed[11,16].

Recently, the use of Power Doppler methods and of oral (polyethylene glycol, PEG) and/or intravenous (Levovist) contrast media, have been suggested to improve the diagnostic accuracy of US, particularly in discriminating inflammatory from fibrotic strictures and in better defining the presence of internal fistulas[27]. These observations are promising, but still too preliminary to suggest the use of these techniques in routine clinical practice.

COMPUTED TOMOGRAPHY

Since the first studies suggesting its possible role in the management of Crohn's disease patients[28], Computed Tomography (CT) has usually been utilized for the detection of extra-enteric complications of Crohn's disease, mainly intra-abdominal abscesses, but is also suitable in the evaluation of strictures, prestenotic dilatations and fistulas. Non-enhanced CT scan is also used in the diagnosis of post-surgical complications (intra-peritoneal abscesses, anastomotic deiscence, extra-abdominal abscesses and fistulas, incisional hernias, ascites, volvolus, bowel adhesions, *etc*.).

This diagnostic technique has also evolved more recently in contrast-enhanced examination, using intravenous administration of iodine contrast agents, and CT-enteroclysis, that can be obtained by means of a fairly large amount (1500 to 2000 mL or more) of contrast agent administered orally or by the positioning of a nasojejunal tube; this technique usually allows the evaluation of the colon as well.

Commonly intravenous and intra-luminal contrast agents are used in combination[29-31]. Various types of intra-luminal contrast media were used to allow negative or positive contrast between the intestinal wall and the lumen[29-35]. Negative intra-luminal contrast agents facilitate the demonstration of normal and diseased bowel segments, particularly after intravenous contrast administration[36].

As a bowel distension media, some authors used a combination of water and iodined contrast agent, or pure water alone. Some authors prefer methylcellulose-water[34]; the latter solution requiring a semi-automatic injector pump.

An appropriate patient preparation with intestinal cleaning should be performed; the small bowel tract should be clean and empty, with lumen distension[32,37,38], avoiding collapsed loops that may mimic wall thickening, strictures and enlarged lymph nodes or abscesses, that can result in diagnostic mistakes[30,33,34].

The intravenous injection of an antiperistaltic drug immediately prior to scanning, blocking the peristalsis, minimizes bowel movement or contraction and allows better intra-luminal distension avoiding the progression of the enteral solution.

A recent study demonstrated that noninvasive peroral CT evaluation of the small bowel is as accurate as CT with jejunal infusion in detecting active small bowel inflammation in Crohn's disease patients[39].

The main findings at CT scan observed in Crohn's disease patients, are small bowel wall stratification and/or thickening ("target" or "double halo" appearance), with or without contrast enhancement, oedema of the mesenteric fat, engorged ileal vasa recta ("comb sign"), sub-mucosal fibro-fatty infiltration and mesenteric adenopathy. The pattern and length of mural contrast enhancement should be carefully evaluated. Wall enhancement is a direct expression of trans-mural inflammation; on the contrary, a thickened non-enhancing tract is usually the result of the evolution in submucosal or trans-mural fibrosis. The limited spatial resolution of conventional helical CT images results in lower rates of demonstration of early disease manifestations and of fistulas and sinus tracts, when compared with enteroclysis examinations.

More recently, the development of multislice helical CT scanners improved visualization of the small bowel[40-42], and abdomen in general, both with higher spatial and temporal resolution acquiring isotropic voxel slices in a single breath-hold scan. These examination modalities allow better three dimensional reconstruction methods (multiplanar reconstruction, volume rendering and surface

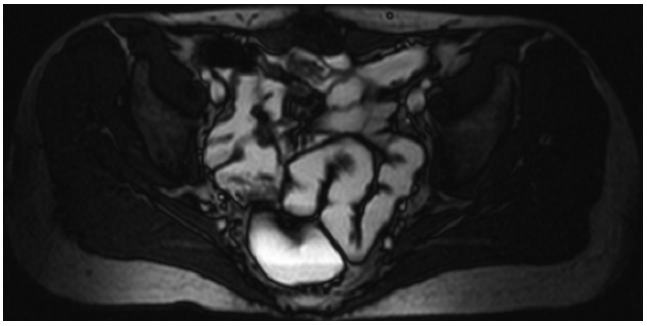

Figure 2 Magnetic resonance, axial T2-weighted fast spin-echo. Mean age female patient with recurrent abdominal pain. This image shows homogeneus small bowel loops distention and thin wall, thus negative for Crohn's disease.

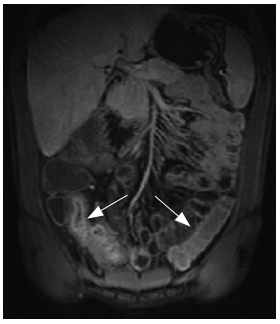

Figure 3 Magnetic resonance, coronal 3D T1-weighted gradient-echo, after intravenous administration of gadolinium contrast agent. Young male patient with known Crohn's disease, recurrent abdominal pain in MR staging of the disease before therapy. The straight white arrow shows the last segment of small bowel with thickened wall and typical selective submucosal enhancement in the arterial phase. The curved white arrow shows a normal intestinal segment with thin wall and no contrast enhancement.

shading display) and volumetric-through navigation (the so called virtual CT-endoscopy).

When compared to endoscopy, small bowel enteroclysis and surgery in the evaluation of small bowel inflammation, CT shows a sensitivity ranging between 71% and 83% and a specificity between 90% and 98%[43,44]. Whether CT scan is also able to assess disease activity in Crohn's disease patients, remains unclear.

In any case, CT plays a relevant role in the acquisition of additional information on extra-luminal complications (mainly intra-abdominal abscesses) and extra-enteric abnormalities[43].

Also, CT represents the standard technique to guide abscess drainage when ultrasound guided drainage is not possible[44].

MAGNETIC RESONANCE

Magnetic resonance (MR) imaging is a non-invasive, non-ionizing radiation diagnostic technique able to obtain multiplanar diagnostic information about intra- and extra-mural involvement of the small bowel by Crohn's disease and its complications[45-47].

MR evaluation of the small bowel had no clinical application and was still considered an experimental technique until the late Nineties mainly because of poor quality in depicting the gastrointestinal tract. Later, MR technical improvements (multichannel and phased-array coils, faster gradients; fast and ultrafast gradient echo and steady state sequences) allowed a deeper insight into luminal and extra-luminal structures with higher spatial resolution during breath-holding.

Small bowel distension with intra-luminal contrast agent had been known for years in conventional X-ray enteroclysis with barium solution, when it was first reported in publications relating to MR imaging[48-50]. Nowadays, this approach should be considered mandatory in order to obtain bowel loop separation and good contrast resolution between lumen, wall and extra-mural structures.

The first examples of new sequences and bowel distension were reported in the late 1990s[51], comparing spin-echo unenhanced sequences with spoiled gradient-echo images with intra-luminal barium contrast agent and intravenous Gd-base paramagnetic contrast, obtaining greater diagnostic value in identifying small bowel wall abnormalities.

Nowadays, thanks to powerful gradients MR, there is a wide use of fast spin-echo and fast gradient echo sequences that are able to acquire 20-30 slices during a single breath-hold. Spectral fat saturation can be added to T2-weighted sequences to maximize contrast between lumen and wall and between bowels and mesenteric fat[52].

Intra-luminal contrast agents can be administered orally or by nasojejunal catheter (NJC). Contrast media are commonly classified as positive, biphasic or negative, according to their action on signal intensity[53]. Oral administration is obviously better tolerated than the positioning and introduction through an NJC. However, the latter technique allows better distension of the small bowel loops because of higher injection pressure in a shorter time.

The necessity to minimize bowel movements during the acquisition is satisfied using an antiperistaltic drug (usually, joscine N-butylbromure) administered intravenously when the patient is positioned inside the MR unit. The use of intravenous paramagnetic contrast agent is a powerful tool to detect the precise localization of the disease[51], or to evaluate the persistence of inflammation despite active therapy[54,55]. Moreover, it also facilitates the diagnosis of mesenterial abscesses, fistulae, and other abdominal or extra-abdominal complications. Gd-chelates are commonly used, followed by 10-20 mL of saline flush.

Arterial and later acquisition phases can be important to distinguish the submucosal enhancement in the arterial phase, that is a distinctive diagnostic sign of active disease in an intestinal segment, especially if the wall is locally thickened. The optimal contrast enhancement can be achieved using fat-saturated sequences.

Importantly, a good correlation between the degree of wall enhancement after intravenous contrast injection and disease activity, calculated using the Crohn's Disease Activity Index (CDAI), has been obtained, with an overall high specificity for MR findings[56]. Several recent studies have shown that MR enteroclysis is characterized by high sensitivity and specificity (> 80%) in the diagnosis and evaluation of Crohn's disease[57-59] (Figures 2 and 3).

VIDEO CAPSULE ENDOSCOPY (VCE)

The introduction in clinical practice of VCE has made available, for the first time in history, a potentially safe and painless endoscopic method to evaluate the entire small bowel.

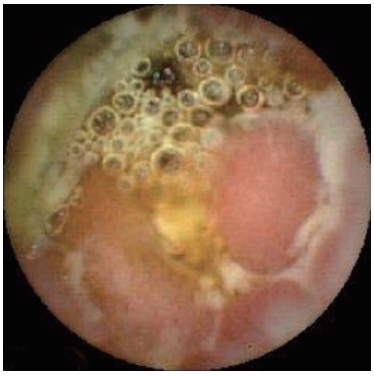

Figure 4 Capsule endoscopy. Multiple ulcers of the terminal ileum leading to lumen sub-stenosis.

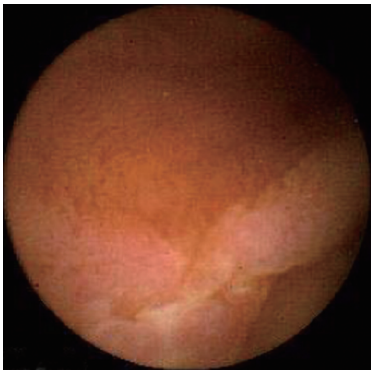

Figure 5 Capsule endoscopy. Linear ulcer of the jejunum.

Table 4 Incremental yield of capsule endoscopy over the other modalities in patients with suspected Crohn's disease (from Triester SL *et al*[63])

	Yield of capsule endoscopy (%)	Yield of other modalities (%)	% Incremental yield for capsule endoscopy (95% CI)	*P*
vs Small bowel radiography	43	13	24 (-0.3-0.51)	0.09
vs Ileoscopy	33	26	7 (-0.12-0.25)	0.48
vs CT enterography	70	21	40 (-0.03-0.83)	0.07

CT: Computed tomography.

Table 5 Incremental yield of capsule endoscopy over the other modalities in patients with established Crohn's disease (from Triester SL *et al*[63])

	Yield of capsule endoscopy (%)	Yield of other modalities (%)	% Incremental yield for capsule endoscopy (95% CI)	NNT	*P*
vs Small bowel radiography	78	32	51 (0.31-0.70)	2	< 0.001
vs Ileoscopy	86	60	26 (0.08-0.43)	4	0.002
vs CT enterography	68	38	30 (0.12-0.48)	-	< 0.01

CT: Computed tomography; NNT: Number needed to treat.

Since its introduction and on the grounds of many papers published in the last five years, VCE has quickly become an essential diagnostic tool in many disease conditions involving the small bowel, although, at the beginning, Crohn's disease was considered a contraindication for VCE due to the risk of asymptomatic strictures potentially leading to capsule retention.

However, the first suggestion of the possible role of VCE in diagnosing Crohn's disease came from the unexpected findings of small bowel lesions (observed with a frequency of about 10%) which were suspected for Crohn's disease (Figures 4 and 5) in several series of patients who had been evaluated for obscure gastrointestinal bleeding[60-63]. Most of these patients only had obscure gastrointestinal bleeding with no symptoms of Crohn's disease and had been thoroughly evaluated with an extensive endoscopic and radiologic diagnostic work-up before performing VCE. Thus, the presence of these unexpected findings at VCE suggested its superiority in detecting Crohn's disease lesions in patients with mild clinical suspicion and negative traditional imaging studies. Indeed, in this clinical setting, VCE appears to be superior to the other, most frequently used, diagnostic techniques (SBE, SBFT, retrograde ileoscopy, entero-CT and entero-MR) although a recent meta-analysis failed to show statistically significant differences in the diagnostic yields of VCE versus other imaging techniques[63-71] (Table 4).

A recent, single-centre, prospective study[72] evaluated the accuracy of VCE in diagnosing Crohn's disease in a cohort of patients with suspicion of the disease (defined as diarrhoea of more than three months duration accompanied by anaemia, and/or weight loss, and/or fever, and/or extra-intestinal manifestations) after 21 mo of follow-up. This study showed high sensitivity, specificity and positive and negative likelihood ratio (93%, 84%, 5.8 and 0.08, respectively) of this new diagnostic tool in diagnosing Crohn's disease.

The role of VCE has also been assessed in patients with known Crohn's disease. Several recent studies[63,73-78] highlighted the superior diagnostic yield of VCE (ranging from 61% to 77%) when compared to that of SBFT, SBE or entero-CT (ranging from 19% to 26%) in assessing small bowel involvement in patients with a previous diagnosis of Crohn's disease. In this setting, VCE appears to have a superior capability in evaluating the extent of small bowel involvement when compared with push enteroscopy[76,78] (Table 5).

There are, however, some limitations to take into account in evaluating these data. In patients with clinically suspected Crohn's disease with no further evidence of the disease, one should be cautious in interpreting VCE findings. In fact, the significance of isolated lesions or mucosal breaks in the small bowel is not clear, since they may be observed also in normal subjects[79], and more frequently in subjects taking NSAIDs[66,79]. Thus, before giving a diagnosis of Crohn's disease to a patient with VCE small bowel lesions only, a careful and thorough clinical evaluation should be performed.

Performing VCE may be helpful in evaluating patients with indeterminate colitis (IC). Indeed, in some series[80,81], 20%-50% of IC patients are characterized by the presence of small bowel lesions, leading to a change in their

Table 6 Pros and cons of the different imaging techniques in the study of the small bowel in Crohn's disease

	PROs	CONs
Bowel ultrasound	-Non invasive, safe and well accepted -Widely available -Information about gut wall and extra-intestinal structures	-Operator dependent -False negative in case of superficial and rare lesions
Conventional radiology	-Exact anatomic location and extent of the lesions	-Limited information about trans-mural and peri-intestinal abnormalities -Radiation exposure
Entero MR	-Information about gut and extra-intestinal structures -Identification of active inflammation -Multiplanar sequences	-costly -Impossible to enter the magnet -IV infusion
Entero CT	-Information about gut and extra-intestinal structures -Mulitplanar sequences	-Radiation exposure -IV infusion -False negative in case of superficial and rare lesions
VCE	-Allows the complete evaluation of the small bowel -High diagnostic yield -Useful in indeterminate colitis -Well tolerated	-Unfeasible if significant stricture present -Not well established specificity of VCE findings
Double-balloon	-Allows the complete evaluation of the small bowel -Therapy and biopsies are feasible	-Invasive procedure requiring sedation and fluoroscopy -No data

diagnostic definition.

Further possible applications of VCE have been suggested in a paediatric setting[82], in the evaluation of post-surgical recurrence[64] and in the surveillance of mucosal healing after biologic therapy[64].

The major limitations of the use of VCE in Crohn's disease are represented by an incomplete evaluation of the small bowel, occurring in 20%-35% of patients[83,84], and by the possibility of capsule retention, occurring in 1%, 4%-6%, 7% of patients with Crohn's disease[85]. To avoid this complication, it is debated whether or not radiological study should be performed before VCE[85]. Further studies are also needed to clarify the possible role of a time-controlled dissolving capsule to be performed before VCE[86-88].

DOUBLE BALLOON ENDOSCOPY

Double balloon enteroscopy (also called push and pull enteroscopy) was described for the first time by Yamamoto *et al*[89] in 2001.

The principle of the double balloon technique allows not only a complete endoscopic evaluation of the small bowel but also makes it possible to take biopsies and to carry out therapeutic interventions along the whole small bowel[90,91]. However, even though double balloon enteroscopy appears a safe and useful technique, when compared with VCE it results in an inconvenient and invasive procedure requiring specialized equipment, sedation of patients, fluoroscopy and prolonged examination time.

Preliminary data suggest its possible use in the diagnosis of Crohn's disease[90,91]; however, to date, there are no published studies comparing double balloon enteroscopy with capsule endoscopy or other imaging modalities in the diagnosis of small bowel Crohn's disease.

CONCLUSION

In recent years, several radiologic and endoscopic techniques have been developed for the study of the small bowel. Each of these techniques is characterized by its own profile of favourable and unfavourable features. The main pros and cons of the different techniques used for the evaluation of the small bowel in Crohn's disease are summarized in Table 6.

Ultrasound and entero-MR appear to be particularly interesting, since neither of them use ionizing radiation. The main advantage of MR 'enteroclisys' over ultrasound is the panoramic view over the whole abdominal cavity, allowing the detection of disease involvement in other gastrointestinal segments or abdominal organs. Moreover, ultrasound is an operator-dependent technique and its use is limited in patients with a large abdomen[12]. On the other hand, ultrasound is a low cost and easily available diagnostic tool.

Thus, we believe that when the diagnosis of Crohn's disease is established and we want to investigate the small bowel, one of these two tests should be performed, and the choice should be based on the presence and availability of the techniques and of experienced operators in the area.

A different choice may be performed when the diagnosis has to be reached, particularly in patients suspected of Crohn's disease and negative upper and lower endoscopy. In these cases, endoscopy appears to be superior to all other techniques, particularly when minute lesions are suspected; thus, capsule endoscopy (when no suspicion of a significant stricture exists) or double balloon enteroscopy should be the preferred tests to be performed. In cases of only isolated lesions and in the absence of a typical histological picture, one should be cautious before making a diagnosis of Crohn's disease on the basis of these findings only.

In general, when we care for patients with Crohn's disease we should always keep in mind that, during the natural course of the disease, these patients will undergo repeated examinations in their follow-up: thus, our goals should always be (1) to perform tests only when they are expected to provide important clues for the management of the patients and (2) to choose those tests with the best profile of diagnostic accuracy and safety.

Keeping this in mind, we have to recognize that all these new developments have opened a new and exciting area in the exploration of the small bowel.

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COMMENTS

Background

Crohn's disease often affects the small bowel and is characterised by the occurrence of several intra- and extra-luminal complications. The study of the small bowel is mandatory in patients with Crohn's disease. In the last years, several techniques have been proposed for the imaging of the small bowel as possible alternatives to the traditional ones.

Research frontiers

To point out the most recent advances in the study of the small bowel in patients with suspected or proven Crohn's disease.

Innovations and breakthroughs

To critically evaluate the advantages and the disadvantages of ultrasonography, radiology and endoscopy in the diagnosis and the surveillance of Crohn's disease affecting the small bowel.

Applications

To optimize the use of the several imaging techniques now available when one care for patients affected by Crohn's disease with small bowel localization.

Terminology

To concisely and accurately describe, define or explain the specific, unique terms that are not familiar to the majority of the readers, but are essential for the readers to understand the article.

Peer review

Valuable review of investigational techniques for Crohn's disease.

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TOPIC HIGHLIGHT

Paolo Gionchetti, MD, *Series Editor*

Ileal pouch surgery for ulcerative colitis

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Abstract

Ulcerative colitis (UC) is a relapsing and remitting disease characterised by chronic mucosal and submucosal inflammation of the colon and rectum. Treatment may vary depending upon the extent and severity of inflammation. Broadly speaking medical treatments aim to induce and then maintain remission. Surgery is indicated for inflammatory disease that is refractory to medical treatment or in cases of neoplastic transformation. Approximately 25% of patients with UC ultimately require colectomy. Ileal pouch-anal anastomosis (IPAA) has become the standard of care for patients with ulcerative colitis who ultimately require colectomy. This review will examine indications for IPAA, patient selection, technical aspects of surgery, management of complications and long term outcome following this procedure.

Key words: Ulcerative colitis; Ileal Pouch; Ileal pouch anal anastomosis

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INDICATIONS FOR SURGERY

Acute

Colectomy most often follows failure of medical treatment for severe and extensive colitis. Toxic dilatation (colon > 6 cm), perforation and haemorrhage are less common indications. The decision to operate is taken jointly and involves daily communication between gastroenterology and surgical teams. Patients receiving high dose intravenous steroids who have a stool frequency of > 8 per day on the third treatment day are likely to require colectomy[1]. Similarly those with a stool frequency of 3-8 stools per day who have a CRP > 45 mg/L are unlikely to settle. Failure to respond after 5-7 d or any significant deterioration during this period is an indication for colectomy. Patients who initially respond but promptly relapse with the reintroduction of diet are also likely to require colectomy. Pouch surgery should be avoided in the acute setting. It is customary to instead, perform subtotal colectomy with an end ileostomy. The colon is mobilized and vessels taken relatively close to the bowel wall. The sigmoid stump is stapled and left long allowing it to be secured with sutures in the subcutaneous space at the lower pole of the wound. Any stump dehiscence will then result in an easily manageable fistula rather than a pelvic abscess and the sigmoid will be easy to locate at reoperation. A Foley catheter is used to decompress the rectum for a period of 3 or 4 d.

Chronic

Large bowel malignancy is ultimately thought to complicate UC in 5% of cases. Meta-analysis has estimated that 2% of those with colitis develop cancer at 10 years, increasing to 8% at 20 years and 18% at 30 years[2]. Magnitude of risk may be decreasing secondary to the effects of screening, prophylactic surgery and adoption of maintenance anti-inflammatory therapy[3]. Nonetheless a family history of colorectal cancer[4] and pan-colitis[5,6] currently mark subjects as high risk. Frequency and severity of relapse are also considered significant factors[7]. Those with PSC are at highest risk of colorectal cancer[8].

Discovery of dysplasia in large intestinal mucosal biopsies provides the best surrogate measure of malignant transformation. Patients judged to be at high risk are subject to colonoscopic surveillance with the aim of detecting dysplasia[9]. Dysplasia associated with UC is microscopically classified as either low (LGD) or high (HGD) grade depending upon the degree of cytological and architectural disturbance. Endoscopic classification defines lesions as flat or raised with further subdivision of raised lesions according to their macroscopic appearance. Raised areas resembling conventional adenomas but situated within an area of colitis are designated as adenoma-like lesions or masses (ALMs). These pedunculated or sessile polyps are usually amenable to endoscopic resection[10]. Areas that demonstrate pronounced irregularity are termed dysplasia-associated lesions or masses (DALMs). These include plaques, velvety patches, areas of nodular thickening and broad

based masses. Such lesions are typically not endoscopically resectable in their entirety.

The vast majority of UC related lesions are macroscopically visible, especially following indigo carmine dye-spray[11]. Complete local excision and surveillance yields a good prognosis, irrespective of the degree of dysplasia. Continued surveillance will identify further ALMs in 50%-60% of patients with flat dysplasia arising in only a small proportion (< 5%)[10,11]. DALMs are usually more challenging to endoscopically remove due to their irregular morphology but in cases where local resection is achieved with clear margins this may be all that is required[12]. Endoscopic assessment of the whole colon must be achieved by an experienced practitioner with facility to use dye-spray techniques in order to uncover otherwise 'occult' colonic lesions[11]. Indications for proctocolectomy following the discovery of a dysplastic mass in our practise are (A) incomplete excision of that mass or (B) discovery of multifocal flat dysplasia of any grade at sites either near to or remote from the index lesion. Biopsy samples must be taken beyond the perimeter of a sessile mass to uncover patients who possess a wider field change. The incidence of underlying malignancy in those who undergo proctocolectomy for DALM is in the order of 30%-40%[12].

The finding of HGD in otherwise flat mucosa is an indication for proctocolectomy as the risk of underlying malignancy is in the order of 40%[13]. This is a relatively unusual finding as isolated HGD is more often associated with some form of discernable lesion. Management of LGD in the absence of a macroscopic lesion is more controversial as its natural history is still hotly debated. It should be appreciated that there is significant inter-observer variability in the reporting of LGD even amongst experienced gastroenterological histopathologists[14]. One problem is that biopsies taken from regenerative mucosa following an exacerbation of UC may be mistaken for LGD. Some institutions favour immediate proctocolectomy for LGD based upon studies demonstrating a 20% risk of occult malignancy at presentation with 50% disease progression in 5 years[13]. We favour a more conservative approach that consists of intensified surveillance with colonoscopy at 6-monthly intervals even in cases of multifocal flat LGD. We believe that thorough endoscopic examination by an experienced clinician obviates the need for routine colectomy for LGD. This strategy has been safely adopted in specialist centres with rates of disease progression between 3% to 10% at 10 years[14,15].

CHOICE OF OPERATION

Three operative strategies are in common use for the definitive surgical treatment of UC patients. (1) Proctocolectomy and end ileostomy removes all diseased tissue at the expense of a permanent stoma. This option is undertaken in patients with poor sphincter function. It is also used in those patients who are happy with their ileostomy following subtotal colectomy and do not wish to consider a pouch. (2) Subtotal colectomy and ileorectal anastomosis (IRA) is a compromise procedure in which a minimally diseased rectum is retained. The rectum must be distensible and retain its capacity to act as a reservoir. This can be confirmed using flexible sigmoidoscopy or a contrast enema. There should be no evidence of colonic dysplasia or malignancy. These criteria are seldom met and this option is rarely used. Function is difficult to predict following IRA when one quarter of patients suffer from unacceptable stool frequency as a consequence of persistent rectal inflammation. Long-term endoscopic follow up of the retained rectum is essential due to the risk of malignant change. (3) Finally ileal pouch-anal anastomosis (IPAA) has become the standard of care for patients with ulcerative colitis who ultimately require colectomy. This procedure was initially developed by Parks and Nichols during the 1970's[16]. They combined elements of Kock's continent pouch[17] with a technique of rectal mucosal excision, used for the removal of rectal adenomata and haemangiomata[18,19]. Their ileal pouch reservoir was anastomosed to the dentate line using a per-anal suturing technique[16]. In a relatively short period of time this technique had become the preferred surgical option for treatment of UC. The advent of stapling instruments greatly simplified IPAA surgery, but it remains a complex undertaking with the potential to cause significant morbidity[20]. This approach is popular with patients as it avoids the necessity for a long-term stoma. Pouch surgery aims to deliver 5 or 6 semi-formed bowel motions per day, with no night time evacuation and no incontinence. Successful outcomes are built upon sensible patient selection, clear pre-operative counselling, an operative strategy appropriate to the patient and expedient management of any complications.

PATIENT SELECTION FOR ILEAL POUCH SURGERY

Age

'Elderly' sphincters were initially considered too weak to undergo the prolonged anal dilatation necessary for mucosectomy and per anal suturing of the IPAA. Introduction of the 'double stapled' IPAA technique meant that prolonged anal dilation could be avoided and reports emerged of IPAA in the 50-70 age group[21-26]. Delaney *et al*[27] found no difference in daytime stool frequency (5-6 stools per day) in 1410 patients < 45 years, compared to 485 over this age with a median follow up of 4.6 years. Nocturnal frequency was a little better in younger subjects (mean 1.4 versus 1.93) during the first year. At one year, episodes of incontinence were reported by one quarter of those below 45 and half of those > 55 years. Night time seepage occurred in one third and one half of patients respectively. Farouk *et al*[28] found that nocturnal stool frequency, faecal incontinence, protective pad usage and consumption of constipating medication were higher in patients aged 45 or more at the time of IPAA. Pouch function deteriorated over time in older but not younger patients. Nonetheless high levels of satisfaction were achieved amongst older patients despite inferior functional results. In summary, surgical complications and pouch preservation rates appear to be independent of age at operation, whilst continence and quality of life are

generally a little worse with advancing years. IPAA surgery is routinely performed in well motivated elderly individuals without symptomatic disturbance of the anal sphincters.

Indeterminate colitis

A definitive histopathological diagnosis of UC or Crohn's is not always possible following colectomy for colitis. In 10%-15% of surgical specimens a diagnosis of indeterminate colitis (IndC) is made[29-31]. Differentiation between UC and Crohn's is usually made difficult by the presence of severe inflammation. For example transmural ulceration in fulminant UC may mimic that normally associated with Crohn's disease[32]. Examination of pre-operative biopsy specimens may yield an accurate diagnosis. Alternatively, the behaviour of the retained rectum may be followed. In UC, florid inflammatory changes are typical while in Crohn's the rectum will tend to improve following diversion[33-36]. Appendiceal orifice inflammation sometimes termed the appendiceal 'skip lesion' can be an additional source of confusion[37-39]. This is considered to be a normal variant of UC being found in 24/94 patients (26%) with active subtotal ulcerative colitis[40].

A diagnosis of Crohn's disease will subsequently be made in 4% to 15% of patients initially labelled as IndC[41]. Clinicians make every effort to define this population prior to embarking upon ileal pouch surgery. While the majority of patients with IndC obtain good results from IPAA surgery, pelvic sepsis and pouch failure may occur more frequently. This is largely due to the emergence of patients with Crohn's disease. At 10 years 85% of those with IndC retain their pouch. The issue of outcome following IPAA for IndC has been addressed in two major studies. The Mayo Clinic compared outcome after IPAA for patients with IndC (n = 82), versus UC (n = 1355)[41]. More Crohn's disease emerged in those with IndC (15% *vs* 2%); median follow-up of 7 years. As a consequence pouch failure was significantly higher for the IndC group (27% *vs* 11%; $P < 0.001$). Outcome in patients with IndC who did not convert to Crohn's was similar to those with UC; although more non-Crohn's IndC patients did manifest pouch fistulas. 85% of pouches were retained at 10 years. Pre-operative features most associated with a subsequent diagnosis of Crohn's were atypical disease distribution such as skip lesions and rectal sparring. The Cleveland Clinic reported more encouraging results but over a period of just 3 years[42]. A post-operative pathological diagnosis of IndC was recorded in 171/1911 IPAA patients (9%). Pouch failure rates were 3% for both UC and IndC. Conversion to Crohn's occurred in 4% of IndC versus 0.4% of matched UC controls. While daytime stool frequency was equivalent (6 ×), those with IndC had worse night time frequency (2 × *vs* 1 ×) and proportionally more soiling (36% versus 28%). Rates of daytime incontinence did not differ (25% moderate, 1% severe). There was less overall satisfaction with pouch surgery amongst patients with IndC although 93% declared that they would undergo surgery again.

The consensus amongst most surgeons is that patients with bona fide IndC are suitable candidates for pouch surgery if fully informed of the risks involved. Special attention should be paid to any suspicious history of pelvic sepsis or perineal fistula as these patients are more likely to manifest Crohn's and in our opinion should not be considered for IPAA surgery.

Crohn's colitis

Following ileal pouch surgery for UC a number of patients are found to have Crohn's disease. The Toronto group reported on 20 such cases from a total of 551 (3%)[43]. 11/20 patients (55%) eventually lost their pouch. Unsuspected Crohn's disease is a leading cause of pouch failure in several other series[44,45]. Following diagnosis of Crohn's disease, pouch failure rates increased 9-fold over a baseline figure of 4%[46]. Pouches were generally lost due to unacceptable function or the presence of complex fistulas. It should also be noted that those with Crohn's who retained the pouch had satisfactory function. A controversial study detailing ten year follow-up of 41 patients with either known colonic Crohn's but no pre-operative perianal or small bowel disease (26/41) or histological features suggestive of Crohn's following panproctocolectomy and IPAA (15/40) reported comparatively favourable results[47]. Early post-operative complications occurred in one quarter, chronic perianal problems in one quarter and pouch failure in 3/41. Function was generally good. Complication rates were proportionally much higher where the diagnosis of Crohn's was unequivocal and controversy exists in cases where minor pathological criteria are used to establish the diagnosis of Crohn's. Some argue that this subgroup might be more appropriately labelled as indeterminate. Crohn's disease remains an absolute contraindication to IPAA for most practitioners as overall failure rates approach 50%. There may be a role for pouch surgery in a highly selected group of patients with Crohn's colitis who possess a normal anus, have no small bowel disease and are prepared to accept the increased risks of failure and reoperation.

Dysplasia or cancer in the proctocolectomy specimen

The presence of dysplasia or potentially curable cancer either within the colon or high in the rectum does not preclude IPAA[48,49]. Mucosectomy and a hand-sewn pouch-anal anastomosis rather than stapling are considered for patients with multiple tumours or multifocal dysplasia especially when these lesions encroach upon the rectum. Following mucosectomy dysplastic cells may survive deep within the muscular rectal cuff[50,51] and these may re-present as 'pouch tumours'[52]. For this reason reconstructive pouch surgery is probably inadvisable when dealing with low rectal tumours.

TECHNIQUE OF ILEAL POUCH SURGERY

Pouch design

Arks and Nicholls originally devised a triple limb 'S' shaped pouch[16]. This was relatively complicated to construct and suffered from kinking of the efferent limb if this was left too long[53]. Alternative designs have included the high capacity 'W' pouch, the H pouch and the 'J' pouch. Lewis *et al* examined factors associated with good functional

outcome in S, J and W double stapled pouches in 100 patients[54]. Compliance of the ileal reservoir, a strong anal sphincter and intact anal reflexes correlated with good outcome while pouch design played no part. The majority of surgeons now favour the J pouch due to ease of construction, economical use of terminal ileum and reliable emptying[55]. Functional results are equal to those of other reservoir designs[56-58]. The pouch is formed from the terminal 40 cm of ileum using several applications of a linear, cutting stapler to join the antimesenteric borders of two 20 cm ileal limbs.

Mucosectomy versus double stapling

Stripping of the columnar mucosa above the dentate line has been advocated in order to prevent recurrence of UC. Mucosectomy, combined with a per-anal hand-sewn anastomosis allows precise placement of the pouch-anal anastomosis at the dentate line. This technique has several disadvantages. It is certainly more complex to perform and may also predispose to higher rates of sphincter damage and incontinence. A study from Cleveland indicated that faecal incontinence was more common after mucosectomy[59]. In the order of 50% of patients will also experience night time soiling[56,60,61]. Mucosectomy entails excision of the anal transition zone (ATZ), an area of cuboidal epithelium richly innervated by sensory nerve endings that mediate anal sampling reflexes (Figure 1)[62]. Two large series have evaluated the effect of ATZ preservation in slightly different ways. Gemlo *et al* audited a change in practise from S pouch combined with mucosectomy to J pouch with double stapled anastomosis[63]. Functional results were reported for 235 pouch procedures. Double stapling was associated with a significant reduction in both major and minor night time incontinence, while minor daytime incontinence was also reduced. Choi *et al* subclassified 138 patients following stapled IPAA according to the epithelial composition of the distal donut[64]. Those with predominantly squamous epithelium (ATZ excised) had significantly lower post operative maximal resting pressures (MxRP) compared to those with mostly columnar epithelium. Values did not however deviate from the normal range. Sphincter length and recto-anal inhibitory reflex preservation did not differ between groups. Surprisingly continence was not reported. These studies tend to suggest that ATZ preservation is a good idea although the role of anal dilation confounds the first study while a lack of functional results hampers the latter.

The 'double stapled' IPAA technique preserves the ATZ with no requirement for prolonged anal dilation. A transverse stapler fired from above, separates the rectum from the top of the anal canal. The stapling instrument should be positioned 2-3 cm above the anal margin, a distance roughly equivalent to the length of the distal 2 metacarpals of the index finger. This helps to avoid an error of judgement that places the anastomosis too high resulting in a pouch-rectal anastomosis. A circular EEA stapler inserted *via* the anus joins the ileal reservoir to the upper anal canal. Proponents of stapling claimed that less sphincter trauma occurs using this technique[65-69] but a series of randomised trials comparing these methods have not demonstrated any functional improvement although some non-significant trends were observed[70-74]. Failure to prove clinical benefit may reflect the small number of patients randomised within each of these trials and the complex nature of defecation.

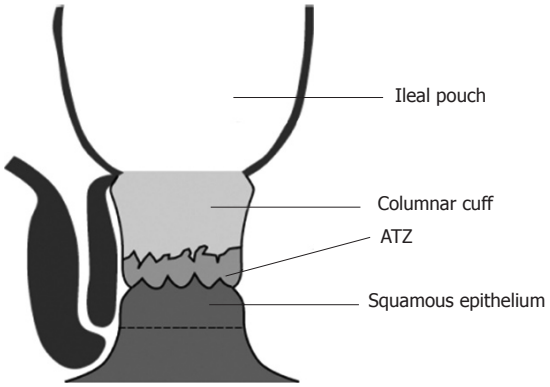

Figure 1 Distribution of epithelial subtypes in a typical double-stapled pouch-anal anastomosis. Reprinted with permission from British Journal of Surgery[119].

Stapling itself is not without risk to the anal sphincter. Winter *et al*[75] reported results of a randomised controlled trial comparing perianal application of 0.2% GTN ointment with placebo in 60 patients prior to circular stapler insertion. GTN significantly reduced intraoperative mean anal resting pressure (MRP) and the need for anal digitation prior to insertion of the circular stapler. Post operative MRP did not deviate from preoperative values and function was excellent at 3 and 12 mo. Following placebo, post operative MRP was significantly reduced and function was worse, even at 12 mo. These findings suggest that local trauma arising from stapler insertion can induce sphincter damage and that pharmacological intervention affords some protection.

Finally, performance of mucosectomy and hand-sewn IPAA is technically more challenging than stapled IPAA. Hand-sewn anastomoses have been associated with higher rates of anastomotic disruption and pelvic sepsis[76] although pouch failure rates are apparently not adversely affected[46]. Many surgeons including ourselves favour the double staple technique as this is the simpler operation; preservation of the ATZ is conceptually appealing and this operation may have a lower risk of morbidity and ultimately failure.

One, two or three stage IPAA

To date most surgeons have favoured creation of a temporary defunctioning loop ileostomy following IPAA surgery as this avoids what can be catastrophic pelvic contamination in the event of anastomotic dehiscence[77]. Pouch failure rates from St Marks were higher in patients without a covering stoma; 15% versus 8%[78], although Toronto have published contrasting figures with less than 1% of one stage pouches failing[43]. To omit a defunctioning ileostomy is an exercise in risk management. Large series indicate that anastomotic separation occurs in approximately 5%-15% of patients[46,79,80] while complication rates for ileostomy closure range from 10% to 30%[81-89]. Small bowel obstruction, wound infection and

anastomotic leakage are the most prevalent. In practice we omit stomas in approximately 15% of cases based upon the perceived risks (steroids, nutrition, age, anaemia etc), uneventful surgery and discharge arrangements.

Laparoscopic IPAA

Conventional open surgery utilises a long midline incision for access to the splenic flexure and pelvis. The laparoscopic approach is more elegant as trauma to the abdominal wall is minimised. In the short term wound related complications such as pain and infection may be reduced. Over a more protracted period the risk of symptomatic adhesions and incisional herniation may be diminished. There is little doubt that cosmetic appearance is enhanced. To date rigorous assessment of these endpoints using large clinical trials has been hindered by the relative complexity of these techniques. A prospective randomised controlled trial of hand-assisted laparoscopic colonic mobilisation and open rectal dissection (*via* an 8 cm Pfannenstiel incision) versus open surgery through the midline in 60 patients showed no difference in post-operative quality of life measurements[90]. While open pelvic dissection is expedient and facilitates distal stapling it may negate some benefits of the laparoscopic approach. Refinement of dissection techniques and the production of dedicated equipment has already greatly facilitated the performance of laparoscopic IPAA in some centres[91]. Accelerated recovery programs have delivered reduced hospital stays for elective IPAA patients somewhat negating the benefits of laparoscopic over open surgery in this regard. In a recent randomised, observer and patient blinded trial, 60 patients underwent elective laparoscopic or open colonic resection with the principles of fast-track rehabilitation applied to both groups[92]. Median postoperative stay was 2 d, with rates of readmission in the order of 20%-25%. More patients thought that their stay was too short following open (30%) versus laparoscopic surgery (17%). Functional outcome did not differ. These data combined with our experience suggest that optimised perioperative management has much to offer the ileal pouch patient.

ACUTE COMPLICATIONS OF IPAA

Acute sepsis

Fever in a patient recovering from IPAA surgery should arouse suspicion of pelvic sepsis. This remains a relatively common acute complication and failure to react in a timely fashion is likely to compromise pouch function and may eventually lead to failure. Septic complications usually result from anastomotic dehiscence or the presence of an infected pelvic haematoma. Digital examination may reveal the anastomotic defect or localised tenderness overlying an indurated or fluctuant mass. CT or MRI can be used to gauge the extent of sepsis. A trial of broad spectrum antibiotics is appropriate for relatively small abscesses. Treatment may be tailored to the size and nature of the problem. For instance non-operative measures were used to treat 24/131 (18%) cases in a series from Heidelberg, with 2/24 (8%) eventually losing the pouch[93]. Data from Mayo indicate that 11/73 (15%) abscesses were considered 'early' and treated with antibiotics alone[94]. All but 3 cases resolved without the need for subsequent surgery. More sizeable collections are considered for radiological drainage and in the series from Mayo an additional 16/73 (22%) cases were aspirated under radiological guidance with only 3 eventually requiring surgical intervention.

Failure to settle would prompt examination under anaesthesia. The anus is inspected using an Eisenhammer anal speculum (Seward, London, UK). Anastomotic breakdown is usually detected without difficulty. The underlying area is then probed to determine the extent of any associated abscess cavity and suction applied to clear its contents. Larger defects may be amenable to digital examination followed by placement of a catheter for irrigation and drainage. Regular re-examination under anaesthetic may be required to be confident that the cavity remains clean. The vagina must also be inspected for evidence of fistulation, especially if the IPAA was stapled. We favour transanal drainage for most episodes of mild to moderate pouch related sepsis though. Other institutions appear to utilise this course of action less frequently with this technique accounting for 8% of treatments for septic episodes in the Mayo series[94] and 33% of those from Heidelberg[93].

At 1 year the rate of pouch related sepsis was 15.6% in 494 consecutive patients treated with stapled J-pouch, mucosectomy and hand-sewn anastomosis from Heidelberg[95]. No patients were diagnosed with Crohn's disease during this period. Fistulae accounted for 76% of septic events (56% pouch-anal anastomotic; 13% pouch vaginal; 7% proximal pouch), with anastomotic separation (16%) and para-pouch abscesses (8%) constituting the remainder[93]. In contrast 73/1508 (4.8%) of patients from the Mayo Clinic had their recovery complicated by a pelvic collection with pouch fistulae recorded in only 3[94]. In this series a technique of hand-sewn ileal J pouch-anal anastomosis was favoured. The Cleveland Clinic evaluated 1965 IPAA procedures performed for UC (60.7%), IC (27.9%), CD (3.8%) and FAP (0.7%) to conclude that fistula formation occurred in 151 (7%), anastomotic separation in 104 (5%) and pelvic abscess in 109 (5%)[46].

Re-laparotomy is reserved for cases where CT guided drainage and minor surgery have failed to control sepsis and also for those who deteriorate quickly with signs of generalised peritonitis. Major leaks require a proximal diverting loop ileostomy to be formed if one is not already in place. Consideration should be given to exteriorising of the pouch if complete anastomotic disruption has occurred. With gross ischaemia one should aim to resect and exteriorise the ileum.

Rates of pelvic sepsis are much higher for patients with UC undergoing IPAA than for those with FAP who are subject to the same operation. High dose corticosteroids (systemic equivalent of > 40 mg prednisolone per day) have been implicated in the causation of anastomotic failure[95,96]. Steroids may impair healing at the anastomosis, promote infection or merely label patients in poor clinical condition. Other series have failed to demonstrate any association between administration of prolonged courses of high dose corticosteroids (> 20 mg) prior to surgery and the rate of acute septic complications[97]. It

is nonetheless customary to avoid IPAA formation and instead perform subtotal colectomy in those patients who are acutely unwell and receiving high dose corticosteroids.

Haemorrhage

Primary intraluminal haemorrhage may follow formation of a sutured or stapled pouch and it is therefore important to carefully inspect the mucosal surface before the pouch-anal anastomosis is constructed. Reactionary intraluminal haemorrhage, within 24 h of surgery is likely to originate from the suture or staple lines. Irrigation of the pouch with a 1:200 000 adrenaline solution controls the majority of clinically significant haemorrhages[20]. Continued bleeding necessitates a return to the operating room. The pouch is inspected using an Eisenhammer speculum, proctoscope or sigmoidoscope. Suction and irrigation are used to accurately locate the bleeding point which is then sutured or injected with 1:10 000 adrenaline solution. Secondary haemorrhage is less common and usually heralds' pelvic sepsis. The pouch should be inspected in theatre with special attention to the ileoanal anastomosis for evidence of localised anastomotic breakdown. Bleeding points are under-run and collections drained, preferably via the original defect. A small mushroom or Foley catheter may then be placed trans-anally into the cavity.

Intra-abdominal haemorrhage may arise from mesenteric vessels or the pelvic side wall. The rectal stump may bleed following hand-sewn pouch-anal anastomosis. In exceptional circumstances inspection of the lower pelvis is facilitated by detachment of the pouch. The stump is approached endoanally using a Lone Star retractor (Lone Star Medical Products Inc, Houston, Tx). The pouch may then be exteriorised as a left iliac fossa mucous fistula if re-anastomosis is considered unsafe. Uncontrollable pelvic haemorrhage requires packing of the cavity with a second look 48 h later.

CHRONIC COMPLICATIONS AND OUTCOME FOLLOWING IPAA

Mucosal adaptation and pouchitis

Prolonged faecal exposure can lead to adaptive changes within the ileal pouch so that it comes to resemble colonic mucosa[98]. The dependent portion of the pouch is most notably affected[99]. Pouchitis is a relapsing, acute-on-chronic inflammatory condition presenting with diarrhoea (that may be bloody), urgency, abdominal bloating, pain or fever. The aetiology is unknown although recurrent UC in areas of colonic metaplasia and bacterial overgrowth are proposed as possible mechanisms. Patients with new symptoms suggestive of pouchitis should be investigated by endoscopy and biopsy. Endoscopic appearances are initially similar to UC. Punctate haemorrhages, mucous secretion, purulent discharge and superficial ulceration occur later. Histological signs of acute inflammation include polymorphonuclear leucocyte infiltration with superficial ulceration, superimposed onto a background of chronic inflammatory changes[100,101]. Interestingly this condition does not seem to affect pouches in patients with FAP.

In a cohort of 123 consecutive 'symptomatic' patients with pouch dysfunction the underlying diagnosis was pouchitis in 34%, irritable pouch syndrome in 28%, unrecognised Crohn's disease in 15% and cuffitis in 22%[102]. Once these disorders have been excluded and a diagnosis of pouchitis is established it would be reasonable to instigate empirical therapy for relapses, with the caveat that patients who do not promptly settle should return for further endoscopic evaluation.

The cumulative probability of pouchitis, determined on the basis of symptomatology, endoscopy and histopathology in 468 IPAA patients was 20% at one year, 32% at 5 years and 40% at 10 years[103]. No pouchitis occurred following surgery for FAP (7% of the total). The incidence of pouchitis appears to be independent of surgical technique with respect to pouch construction, use of a defunctioning stoma or laparoscopic techniques[58,104-106]. Patients with PSC are more prone to develop pouchitis, with a cumulative probability of 79% at 10 years[107]. Persistence of extraintestinal manifestations of UC has also been linked to an increased risk of developing pouchitis and certain patients exhibit a temporal relationship between their pouchitis and extraintestinal symptoms akin to that described for UC, fuelling speculation that these two inflammatory processes represent variations of the same underlying condition[108]. Perpetuating this theme, smoking is considered to be protective against UC[109] and also reduces the incidence of pouchitis[110,111].

First line therapy is with oral metronidazole or ciprofloxacin. Hurst *et al*[112] concluded that oral metronidazole or ciprofloxacin clinically improved 96% of pouchitis in an institutional series from Chicago. 41/52 subjects were successfully treated using a seven day course of metronidazole 250 mg tds, with a further 8 responding to ciprofloxacin 500 mg bd. Two thirds of patients developed further attacks and 6% became chronic sufferers. The efficacy of metronidazole has been confirmed by three small prospective randomised studies[113-115]. One suggested that ciprofloxacin 500 mg bd for two weeks was more effective than metronidazole[115]. This drug produced no side effects whereas metronidazole had induced either an unpleasant taste, vomiting or transient peripheral neuropathy in 3/9 patients. Maintenance therapy may be effective for those who promptly relapse following cessation of treatment and weekly rotation of antimicrobials may combat resistance to single agents. The probiotic VSL-3 may be taken orally with some evidence that relapse rates are decreased. Two randomised trials have shown relapse rates in the order of 10%-15% at 9-12 mo with VSL-3 versus 94%-100% for placebo[116,117]. This therapeutic agent has also been trialled in a prophylactic capacity following IPAA surgery. At one year 10% of VSL-3 patients had experienced at least one episode of pouchitis in contrast to 40% of those receiving placebo[118]. Those who fail to respond may be offered oral or rectal corticosteroids[100]. Alternatively oral or topical mesalazine may be used. Consideration should be given to removing the pouch where function is very poor as a consequence of chronic pouchitis.

Cuffitis

The ATZ forms a relatively small proportion of the anal canal. Conventional double-stapled restorative proctocolectomy leaves 1.5-2.0 cm of columnar epithelium above the ATZ (Figure 1)[119]. Recurrent UC within the columnar cuff is termed 'cuffitis' and it arises in 9%-22% of patients[120,121]. Cuffitis may lead to increased stool frequency, bloody discharge, urgency and discomfort. Mesalazine suppositories may be helpful in improving these symptoms[102]. Dysplasia or carcinoma may theoretically arise within unresected columnar mucosa. Reports do exist of adenocarcinomas situated below the level of the IPAA but these lesions are generally associated with the presence of severe dysplasia or malignancy within the original proctocolectomy specimen[52,122-126]. Routine surveillance of the anal canal is not advocated for the first ten years following IPAA unless the patient has a previous history of dysplasia or malignancy[127-130].

Small bowel obstruction

In a large series from Toronto the risk SBO outside of the perioperative period was reported as 6% at 1 year, 14% at 5 years and 19% at 10 years[131]. One quarter of these patients experienced more than one episode. Laparotomy was required in one third of patients and in the majority of cases small bowel was adherent to the pelvis or a previous stoma site. 20% of patients who underwent laparotomy and adhesionolysis developed further episodes of SBO. One quarter of these had a further laparotomy. Factors predisposing to SBO were revisional pouch surgery and formation of a defunctioning stoma. Bowel ischaemia was a rare finding and so a non-operative strategy is likely to be safe where signs of ischaemia do not exist. A water soluble contrast enema may help to determine the site, nature and degree of obstruction. This investigation may also be of therapeutic benefit. Alternatively CT with oral contrast provides similar information. Separate reports from the Cleveland[20], Mayo[132] and Lahey[133] Clinic's, with follow-up of 2 to 3 years document SBO rates of 25%, 17%, and 20% respectively, with operative intervention necessary in 7% of cases.

Several strategies have been devised to prevent adhesion formation. A multicenter randomised controlled trial of the sodium hyaluronate bioresorbable barrier preparation Seprafilm (Genzyme, Cambridge, MA), revealed reduced adhesions to the midline scar following IPAA in cases where this product was used[134]. Unfortunately the incidence of SBO remained unchanged. If applied next to an anastomosis Seprafilm may impair healing[135], a finding that in our view would preclude its use within the pelvis of pouch patients where adhesions commonly give rise to episodes of SBO.

Chronic pelvic sepsis

Pelvic sepsis is estimated to complicate 10%-20% of IPAA procedures. Long term manifestations of pouch sepsis include a variety of fistulae (pouch-anal anastomotic, pouch vaginal, pouch perineal or proximal pouch) and anastomotic stenosis. Functional outcome is likely to be worse following pelvic sepsis both in terms of frequency, reliance upon constipating medication and incontinence. Long term ileostomy may be required in some. Persistent pouch fistula, poor function secondary to a compromised anal sphincter or outlet obstruction may all contribute towards pouch failure.

Fistulae arising between the IPAA and vagina occur relatively rarely with an estimated incidence estimated of 3% to 16%[61,136-139]. In a study of 68 patients from St Marks pouch vaginal fistulae originated from either the IPAA (76%), the pouch (13%) or from a cryptoglandular source (10%)[140]. Operative trauma, postoperative pelvic sepsis and undiagnosed Crohn's disease were implicated. Unsuspected Crohn's should be actively sought as rates of healing are worse (25% *vs* 48%) and pouch failure more common (33% *vs* 14%) amongst this subgroup[139]. Principals of management include local drainage of the tract using a seton with faecal diversion in selected cases based upon the degree of uncontrolled sepsis. Several options are available to the surgeon for definitive treatment. Transanal ileal advancement flap is appropriate for a pouch that remains mobile with success rates reported in the order of 50%[141]. The procedure may be repeated if it initially fails with some success. Transabdominal advancement of the ileoanal anastomosis with closure of the defect is necessary when the pouch cannot be mobilized from below. Per-anal access to fistulae arising within the anal canal may be difficult, especially where an anastomosis has been placed at the anorectal junction. For this reason the transvaginal route is favoured by some as access is easier and damage to the anal sphincters may be avoided[136]. The internal anal opening is exposed through the posterior wall of the vagina. Following this the pouch is mobilized and the defect closed followed by restitution of the vaginal wall and formation of a defunctioning stoma[142]. Fistulae that arise as a consequence of previously unrecognised Crohn's disease may be treated with infliximab although recurrence remains a problem[143,144].

Anastomotic stricture may complicate leakage, tension or ischaemia at the IPAA[145]. This is estimated to complicate 4% to 18% of cases[20,69,146-148]. It is therefore important to perform an adequate EUA prior to ileostomy closure in addition to the pouchogram. Once the pouch is in circuit symptoms of straining, diarrhoea and anal or abdominal pain suggest stricturing of the anastomosis. It may be possible to attempt dilatation at the time of pouchoscopy. Alternatively application of Hegar's dilators under anaesthesia successfully treats most cases[149]. It may prove beneficial for the patient to continue to use the dilator for several weeks at home. Particularly long or tight strictures may not respond to these measures. Further biopsies are taken to exclude Crohn's disease. Per-anal pouch advancement is considered once all sepsis has been eradicated if the pouch is not tethered[141]. This technique is also used to close fistula tracks situated at the level of the stricture. Otherwise re-laparotomy, mobilisation of the pouch with re-anastomosis is the sole option.

Sexual dysfunction

Erectile function is a parasympathetic response mediated by the erigent nerves, while ejaculation is a sympathetic

event mediated by the hypogastric nerves. These structures may be damaged during pelvic dissection as they lie behind the parietal facial envelope, close to the mesorectal plane. One may avoid contact with the pelvic nerves using a close rectal dissection. This approach is highly vascularised and for this reason many surgeons prefer to dissect in the more anatomical mesorectal plane. Lindsey *et al*[150] deduced that close rectal dissection conferred no benefit with regard to either impotence or ejaculatory difficulties when compared to dissection in the mesorectal plane. Sexual dysfunction affects 3% of men following pouch surgery and for this reason sperm banking should be recommended[28,150]. Sildenafil (Viagra) has been shown to help erectile dysfunction but will not impact upon retrograde ejaculation[151].

Fecundity and pregnancy

UC commonly affects young females of reproductive age. Neither the disease itself nor the medical treatments currently available (apart from salazopyrin in men) are thought to compromise fertility[152]. Fertility rates are lower in women who have had pouch surgery compared to those who undergo purely medical management. In the order of 40% of women will have difficulty becoming pregnant following IPAA[153]. It may be possible to delay proctectomy until a family has been established or alternatively use of anti-adhesion products may combat tubal obstruction.

Vaginal delivery has been associated with occult sphincter injury in 30% of patients[154]. Females with an ileal pouch might risk incontinence following vaginal delivery. Cleveland clinic has reported that sphincter injury occurs more frequently in those who choose vaginal delivery rather than caesarean section with rates of 50% and 13% respectively but no difference in pouch function was apparent at 5 years[155]. The Mayo Clinic reported that pouch function was unaffected by childbirth in 85 women; median follow up of 8 years[28]. For the duration of the pregnancy stool frequency, incontinence and pad usage gradually increase[156]. Pouch function quickly returning to normal in most cases. A study of 47 deliveries in 29 women from Toronto revealed that stool frequency and incontinence were worse in the third trimester with pouch function quickly returning to normal in 83% of cases[157]. Neither multiple births nor birth weight adversely affected subsequent pouch function. Long-term disturbance in pouch function was seen in a small proportion of females (17%) although this interestingly bore no relation to the method of delivery (Caesarian or vaginal). 24/49 deliveries were by Caesarian section. The authors concluded that obstetric criteria alone should determine mode of delivery. It seems reasonable to conclude that while vaginal delivery confers no functional disadvantage in the medium term we remain concerned that sphincter integrity is indeed compromised. Long-term implications remain unmeasured and therefore uncertain.

Pouch failure

Complication rates for IPAA in the order of 30% to 40% are relatively high. Fortunately most of these problems can usually be resolved. Pouch excision or indefinite retention of a defunctioning stoma defines failure. Institutional pouch failure rates have notably fallen over the past 20 years presumably following improvements in patient selection and surgical technique. Long term failure occurs with a frequency of 5%-10%[43,46,78]. A consistent theme that emerges from the large institutional series is that early pouch failure is closely associated with the occurrence of perioperative pelvic sepsis while that occurring later is often secondary to poor function or following an unexpected diagnosis of Crohn's disease[28,43,46,78,103]. Most failures occur beyond the first year and a steady rate of attrition occurs up to 10 years. Certain operative practises, such as the S pouch design, may have increased pouch failure rates in the past.

The success of redo pouch surgery for UC has improved with approximately three quarters of patients now retaining a functional pouch in the long term[158]. This figure rises further when considering patients with isolated functional impairment[159]. When considering revision one should evaluate the sphincters, assess pelvic soft tissue compliance, make a judgement regarding the likely diagnosis (Crohn's or UC) and determine the patient's general health and wishes. It is clear that redo-IPAA surgery may benefit patients with an excessively long efferent ileal spout[159-161] or those with a tortuous stricture[162]. It is perhaps less clear whether revision is as beneficial to those with ongoing septic complications[149,163]. Of 101 pouch revisions performed at the Cleveland clinic the original cause of failure was listed as perineal or pouch vaginal fistula (47%), pouch dysfunction including a long efferent limb (36%), chronic anastomotic leak (27%), anastomotic stricture (22%) or unclassified (6%)[164]. Pathological evidence of Crohn's disease was noted in 4 patients prior to revisional surgery and a further 15 following its completion. New pouches were fashioned in 28 patients with the rest undergoing revision in order to preserve bowel length. Outcome data were available for 85 patients with pouch survival rates at 5 years of 79% for UC and 53% for Crohn's. Continuing sepsis was present in 64% of cases at the time of revision but this did not prejudice the outcome. Stool frequency was 6.3 ± 2.8 by day and 2.0 ± 1.9 at night. Values were higher where a new pouch had been constructed. Faecal seepage occurred in 50% by day and 69% at night. Complications arising as a result of redo-IPAA occurred in 46% of patients. These results indicate that even in the best hands redo-IPAA surgery carries an appreciable morbidity rate. Not surprisingly outcomes are worse both in terms of overall failure and function when compared to first time surgery; nonetheless this procedure remains a valid alternative to a defunctioning stoma or pouch excision.

When faced with the proposition of removing an ileoanal pouch one should consider that 62% of 68 cases treated at St Marks suffered significant morbidity and one patient died[165]. Pouch failure was attributed to sepsis (50%), poor function (35%), pouchitis (8%) or an assortment of other causes. Salvage had been attempted in 82% of cases prior to excision. The single most common complication following pouch excision was non-healing of the perineal

wound with an incidence of 40% at 6 mo and 10% at 12 mo. Between 1 and 6 procedures (median 2) were performed per person to facilitate healing. The risk of readmission at 1 and 5 years was 38% and 58% respectively with 20% of patients requiring reoperation for small bowl obstruction, stoma complications or haemorrhage. A technique of close pouch dissection was used to avoid impotence. Unfortunately 7% of males ultimately suffered from this complication. The success of redo pouch surgery for UC has improved with approximately half to three quarters of patients now retaining a functional pouch in the long term. When considering revision one should evaluate the sphincters, assess pelvic soft tissue compliance, make a judgement regarding the likely diagnosis (Crohn's or UC) and determine the patient's general health and wishes. It is clear that redo-IPAA surgery may benefit patients with an excessively long efferent ileal spout or those with a tortuous stricture. It is perhaps less clear whether revision is as beneficial to those with ongoing septic complications. Even in the best hands redo-IPAA surgery carries an appreciable morbidity rate. Not surprisingly outcomes are worse both in terms of overall failure and function when compared to first time surgery; nonetheless this procedure remains a valid alternative to a defunctioning stoma or pouch excision.

CONCLUSION

The introduction of IPAA has revolutionised treatment of ulcerative colitis. Over the past 30 years we have witnessed convergence of operative technique towards a stapled J pouch design with stapled ileo-anal anastomosis. This is perhaps the fastest and easiest way to create the IPAA. Anastomotic design will hopefully evolve further in order to minimise post-operative complications, reduce the frequency of bowel movements and improve continence. One stage laparoscopic IPAA has already set new standards of cosmesis and may reduce the burden of adhesional small bowel obstruction. Perennial problems such as evolving Crohn's disease still produce substantial morbidity amongst a minority of patients. We look forward to the development of genetic markers that identify this subgroup at an early stage so that pouch surgery may be either avoided or prophylactic therapy initiated to improve outcome. Pouchitis is a more common problem for which we hope that determination of the relevant aetiological factors may allow prophylaxis. Ileo-anal pouch surgery has quickly become the standard of surgical care for chronic UC and should be considered a major success in the field of gastrointestinal surgery.

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REVIEW

Diagnostic procedures for submucosal tumors in the gastrointestinal tract

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Abstract

This review is part one of three, which will present an update on diagnostic procedures for gastrointestinal (GI) submucosal tumors (SMTs). Part two identifies the classification and part three the therapeutic methods regarding GI SMTs. Submucosal tumors are typically asymptomatic and therefore encountered incidentally. Advances in diagnostic tools for gastrointestinal submucosal tumors have emerged over the past decade. The aim of this paper is to provide the readers with guidelines for the use of diagnostic procedures, when a submucosal tumor is suspected. Literature searches were performed to find information on diagnostics for gastrointestinal submucosal tumors. Based on the searches, the optimal diagnostic procedures and specific features of the submucosal tumors could be outlined. Standard endoscppy, capsule endoscopy and push-and-pull enteroscopy (PPE) together with barium contrast X-ray do not alone provide sufficient information, when examining submucosal tumors. Endoscopic ultrasound (EUS), computed tomography (CT), magnetic resonance imaging (MRI) and fluorodeoxyglucose-labeled positron emission tomography (FDG-PET) are recommended as supplementary tools.

Key words: Submucosal tumor; Diagnosis; Endoscopy; Endoscopic ultrasonography; Computed tomography; Magnetic resonance imaging; Positron emission tomography; Capsule endoscopy; Push-and-pull enteroscopy

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INTRODUCTION

A submucosal tumor (SMT) is defined as any intramural growth underneath the mucosa, where etiology cannot readily be determined by luminal diagnostic endoscopy or barium radiography[1].

The incidence of SMTs in the entire gastrointestinal (GI) tract is not known. However, gastric SMTs occur with an incidence of about 0.4% in diagnostic endoscopy[2]. Following the introduction of new diagnostic procedures, e.g. capsule endoscopy, a more accurate incidence may be found within the next years. Final diagnosis is made with immunohistochemistry and electron microscopy as described in part two of this series of reviews.

SMTs are usually asymptomatic and therefore most often discovered as accidental findings during surgery, autopsy or diagnostic procedures. If symptoms do occur, they are unspecific such as abdominal pain, obstruction, hemorrhage and intussusception[1,3-5]. Like other malignancies, malignant SMTs may present with systemic symptoms, especially weight loss[1,4,6].

The aim of this paper is to update the reader on diagnostic procedures, when investigating a lesion suspected to be a SMT in the GI tract.

DIAGNOSTIC PROCEDURES IN SUBMUCOSAL TUMORS

Standard endoscopy

Due to their lack of overt symptoms, SMTs are generally discovered accidentally during standard endoscopic examination. A lumen diminishing process with or without ulcerations is typically seen, but extramural pathology must be considered as a differential diagnosis[2]. Since standard endoscopy is not sufficient for diagnosing SMTs, suspicion of such requires further examination by means of diagnostic procedures mentioned below[7].

Capsule endoscopy

With capsule endoscopy parts of the small intestine

inaccessible to standard endoscopy can be viewed. Its main indication is obscure hemorrhage with negative upper and lower standard endoscopic findings. A period of 8-12 h of fasting prior to the examination is required[8]. The capsule provides approximately 8 h of continuous endoscopic video imaging of the esophagus, stomach, small intestine and right colon. The capsule is wireless, equipped with white light-emitting diodes and has a size of approximately 1 cm × 2.5 cm. It is disposable, propelled by peristalsis and excreted after 24-48 h. There is no need for air inflation of the gut lumen. Data are transmitted employing radiotelemetry to aerials attached to the body. A study typically takes 30-60 min to review. The procedure is safe, painless, does not require sedation, can be performed ambulatory and does not have the risk of perforation as does standard endoscopy[8,9].

With capsule endoscopy, a villus-based view is generated as opposed to the lumen-based view in standard endoscopy. Therefore, tumors may have a different appearance in these two procedures[8]. The capsule cannot wash an area, and it is not possible to re-examine a possible abnormality, take biopsies or deliver therapy as it is with standard endoscopy[8] and PPE[10]. Furthermore, a recent study found a tendency towards poor interobserver agreement for abnormalities in relief (tumors and ulcers), but good for red-colored abnormalities (bleeding and angiodysplasia). However interobserver agreement was significantly better among experienced endoscopists than among less experienced[9].

Occasionally, the capsule is caught in a stricture or diverticulum. A plain abdominal X-ray can be performed to determine whether the capsule is retained or not. However, this often happens at the site of pathology, where surgery is required anyway. Removal of an impacted capsule may be performed endoscopically[8].

Push-and-pull enteroscopy

PPE is an alternative to capsule endoscopy. With PPE the small intestine can be examined using a double-balloon technique with an oral and/or anal approach. Indications include GI bleeding, abdominal pain and surveillance of known disease. The advantage of PPE is that it is relatively safe, has a high diagnostic yield and both biopsy and endoscopic therapy can be performed[10].

Disadvantages include the risk of perforation, the need for conscious sedation and the related complications, and the fact that the latex balloons used create a potential risk of anaphylactic shock in patients with latex allergy[10,11].

Side effects are usually mild, such as abdominal pain for 1-2 d, brief fever, reddening of the mucosa, slight intramucosal hemorrhage in the small-bowel tissue and vomiting after the procedures. Aspiration pneumonia after an epileptic attack induced by the propofol anesthesia was found as the only complication in a recent prospective study of 100 patients[10].

Endoscopic ultrasonography

The tool of first choice for examining SMTs in the upper GI tract is endoscopic ultrasonography (EUS). It is the most accurate procedure for detecting and diagnosing SMTs, due to its high sensitivity and specificity[12-16]. EUS is performed as the second intervention following standard endoscopy[16].

The most important application of EUS is staging of GI malignancies, since this dictates the management and predicts survival of patients[15,17,18]. EUS features suggestive of malignancy are irregular borders, abnormal lymph nodes, ulcer, and a shape that is not oval or round[19]. Heterogeneous echopattern is a feature of controversy[20].

EUS is useful in differentiating between intramural tumors, intramural vascular lesions and extraluminal impressions with or without the use of Doppler-EUS[13,14,21]. EUS can provide information concerning origin, size, borders, homogeneity and foci with echogenic or anechoic features (Table 1)[12,22-24]. In addition, EUS can indicate whether endoscopic resection is appropriate[13,14,25].

In tumors smaller than 0.5 cm, high-frequency transducers can obtain information that is not available even with highly sophisticated CT, magnetic resonance imaging (MRI), transabdominal ultrasound[26] or positron emission tomography (PET)[27]. Intramural abnormalities can be investigated with frequencies of 12 MHz, whereas 7.5 MHz reveals the extramural structures[21]. Its high resolution and the close proximity of the ultrasound probe to the site of the SMT makes EUS valuable in determining the layer of origin of a SMT and the possible invasion of other layers[21].

However, benign SMTs, malignancies and nonneoplastic lesions, such as inflammation, can not be distinguished enosonographically[21,22]. Nevertheless, as EUS is a valuable tool in assessing local lymph node involvement[28], this finding supports the differentiation. A study concerning EUS evaluation of leiomyomas concludes that EUS is quite observer-dependent because the interobserver agreement had a kappa value of only 0.53[29]. Optimally, the same examiner should perform all of the EUS examinations concerning the same patient in order to determine tumor progression versus regression.

Some important tasks of EUS in SMTs are shown in Table 1. EUS criteria for malignancy are outlined in Table 2, to which rapid growth rate found on follow-up can be added[13]. It must however be emphasized that only microscopic examination can determine the final diagnosis and whether the SMT is benign or malignant[19].

Endosonographically, the wall of the GI tract consists of 5 layers of alternating echogenicity (Figure 1). The 1st layer is hyperechoic and represents the superficial layer of the mucosa. The 2nd layer is hypoechoic and constitutes of the deep layer of the mucosa, including the muscularis mucosae. The 3rd, hyperechoic layer is the submucosa, the 4th hypoechoic the muscularis propria and the 5th hyperechoic is the serosa/adventitia[21,22]. As an example, a myogenic SMT can be diagnosed with confidence, if there is continuity between a hypoechoic SMT and the 4th, hypoechoic, layer of the adjacent normal GI tract wall[30].

Catheter probe-endoscopic ultrasonography

Catheter probe-endoscopic ultrasonography (CP-EUS) can probably be used instead of EUS for the evaluation of small SMTs[31]. The concept of CP-EUS is that an ultrasound catheter probe can be inserted through the accessory channel of a conventional endoscope. Thereby

Table 1 Macroscopic and endoscopic ultrasound features of the submucosal tumors

	Endo-scopical	Size	Distinct borders	Ulcer	Layer	Form	Echogenecity		Number	Consistency
Leiomyoma[3,21,22,29,32,63,101-105]	Umbilicated	< 5 cm	Yes	Central or normal mucosa	4th (2nd)	Smooth	Homo	Hypo	-	Firm
Granular cell tumor[4,22,28,60,62,63,106-108]	Yellow	< 2 cm	Mostly no	No	2nd, 3rd, 4th	Sessile polyps, nodules or plaques	Mosaic	Hypo	S (M)	Very firm
Ectopic pancreas[4,13,21,22,31,64,66]	Duct opening	1-4 cm	Yes (no)	No	3rd,4th (2nd, 5th)	Sessile, hemispherical	Perhaps hetero	Hyper	S	Firm
Schwannoma [19,22,28,58,95,109]	Spherical (multi-nodular)	3 cm (0.5-10 cm)	Yes (sometimes fibrous capsule)	No	4th (3rd)	Round/oval (multinodular)	(Homo)	Hypo, bull's eye[5]	S (M)	-
Lipoma[4,13,21,22,29,32,67,110,111]	Yellow	-	Yes, pseudocapsule	Most often intact mucosa, but ulcers do occur	3rd (4th, 5th)	Polypoid, discrete, round	Homo	Hyper	S	Soft, compressible
[3]Neurofibroma [28,50,68,74,112]	Some times long segments of nodular thickening	Few mm. up to a meter	Yes, often macroscopically	No	May involve all layers	Fusiform, diffuse, "ropelike" or "bag of worms"	Hetero	-	M	Rubbery or firm
Vascular[4,13,21,28,29,73,113]	Lymph-angiomas: yellow	Depending on type	Yes	No	2nd, 3rd; cavernous may involve all layers	Round/ oval/ wavy	Homo	An-/hyper[1]	M/S	Liquid/soft
Leiomyosarcoma [21,22,32,43,72,78]	Exophytic	> 3 cm	Irregular	Deep ulcer (> 5 mm)	2nd, 4th	Nodular, polypous	Hetero	An- areas[2]	S	Softer than leiomyomas
Kaposi's sarcoma [4,43,63,85]	Red-purple	Varying (see text)	-	Often ulceration and bleeding	1st, 2nd, 3rd	Maculopapular/ nodular/ polypous	-	-	M/S	-
Metastases[6,22,86,114]	Endo-/ exophytic	-	No	Yes/No	All layers	Volcanolesions, nodules, polyps, linitis plastica	Depends on the primary tumor	-	M/S	-
GIST[4,13,19,22,50,93]	Varying	> 2 cm	Yes/no	Occasionally	4th (2nd, 3rd, 5th)	Elliptical, multilobular/ pedunculated; smooth/nodular	Homo/ hetero[4]	Hypo, bull's eye[5]	S[3]	Friable

[1]Hyperechoic in lymphangiomas[13]. [2]The anechoic areas are histologically consistent with necrotic areas[21]. [3]Neurofibromatosis type 1 is associated with gastrointestinal stromal tumors (GIST). GIST are often multiple in neurofibromatosis type 1[50]. [4]Normally gastrointestinal stromal tumors are homogenic, but if the tumor is large, central necrosis (cystic spaces) can result in heterogeneity[19,44]. Furthermore, echogenic foci and calcifications may be seen[19]. [5]A hypoechoic, marginal halo resulting in a bull's eye appearance of the SMT[19]. Homo: homogeneous; Hetero: heterogeneous; Hypo: hypoechoic; Hyper: hyperechoic; An: anechoic. -: means that no date was found on the subject.

both endoscopy and EUS can be performed during the same intervention[16,31]. The clinician should bear in mind that CP-EUS images tend to be more hypoechoic than EUS images[16].

Due to the small diameter of the CP-EUS probe and the absence of a balloon at its tip, compression of the inner layers is avoided and thus blurring[16]. CP-EUS identifies the layer of origin of myogenic SMTs with great precision and is better than EUS at distinguishing between the two layers of the muscularis propria[16].

In a recent study, CP-EUS diagnosed more than 95% correctly in large intestinal SMTs, confirmed by biopsy or surgical resection[32]. Another study of 25 SMTs, showed that CP-EUS and EUS equally visualized all SMTs, with image quality and determination of tumor diameters and margins being comparable[16]. On the contrary, Chak *et al*[31] found that CP-EUS, but not EUS, staged submucosal lesions correctly in all cases, confirmed by histology. Staging of regional cancer was concordant between EUS and CP-EUS in 80% of the cases. However, these results may be influenced by a selection bias, as only smaller SMTs were chosen for CP-EUS examination.

A shortcoming of CP-EUS is the risk of neglecting other SMTs, since examination occurs only directly at the region of interest[16]. Due to its smaller diameter it could be suspected that CP-EUS would have an advantage in stenosing SMTs that cannot be traversed by an EUS-endoscope. However, stenosing tumors tend to be bulky,

Table 2 Endoscopic ultrasonographic criteria for malignancy in different SMTs

Reference	Tumor type	Criteria for determining the SMT as malignant or borderline	Size	Irregular borders	Abnormal regional lymph nodes	Heterogeneous cho pattern		Shape not oval/round	Ulcer
						Cystic spaces	Echo-genic foci		
Ando *et al* 2002[30]	GIST	Size > 5 cm and at least 1 of the 2 other features:	> 5 cm	Yes	-	Yes	-	-	-
Nickl *et al* 2002[20]	Hypo-echoic SMTs	1 or more of the features:	> 3 cm	Yes	Yes	No	No	Yes	Yes
Brand *et al* 2002[24]	SMTs in general	2 or more of the features or 1 and clinical symptoms (pain, dysphagia, weight loss, hemorrhage)	> 3 cm	Yes	-	Heterogeneous echo pattern		-	-
Rösch *et al* 2002[12]	SMTs in general	2 or more of the features:	> 3 cm	Yes	Yes	Heterogeneous echo pattern		-	-
Palazzo *et al* 2000[23]	GIST	2 or more of the features:	-	Yes	Yes	Yes	-	-	-
Chak *et al* 1997[115]	GIST	2 or more of the features:	> 4 cm	Yes	-	> 4 mm	> 3 mm	-	-

Nickl and coworkers[20] had the highest sensitivity rate (100%) while Palazzo and coworkers[23] had the highest specificity rate (88%). -: means that this feature was not part of the criteria in the given study.

and since CP-EUS has limited depth penetration compared to EUS, CP-EUS may fail to visualize the extraluminal margin and assess adjacent lymph nodes[31]. If the SMT is larger than 5 cm in diameter, EUS or CT may be the preferable imaging techniques[16].

Condom-catheter probe-endoscopic ultrasonography

In the esophagus, the acoustic coupling needed for EUS is impaired by the lack of a water-filled lumen. Therefore a method has been developed specifically for this situation: small-diameter CP-EUS with an attached latex condom (condom-CP-EUS) that can be filled with water[33].

A limitation of condom-CP-EUS, when using large echoendoscopes, is compression of small esophageal tumors and thus distortion of the image[33]. The wall layers are also often compressed, and therefore only 3 layers of the esophageal wall are seen, compared to the 5 layer pattern with 7.5-12 MHz probes[21].

Other shortcomings are limited depth penetration and poor acoustic coupling, resulting in low quality images and impeded evaluation of lymph nodes and bulky SMTs. Moreover, a large volume of water is needed for adequate acoustic coupling, which may leak and cause aspiration. Large SMTs may create air artifacts between the condom and the esophageal wall[33]. Additionally, there is a potential risk of anaphylactic shock, due to latex allergy, which has a prevalence of less than 1% in the normal population[11].

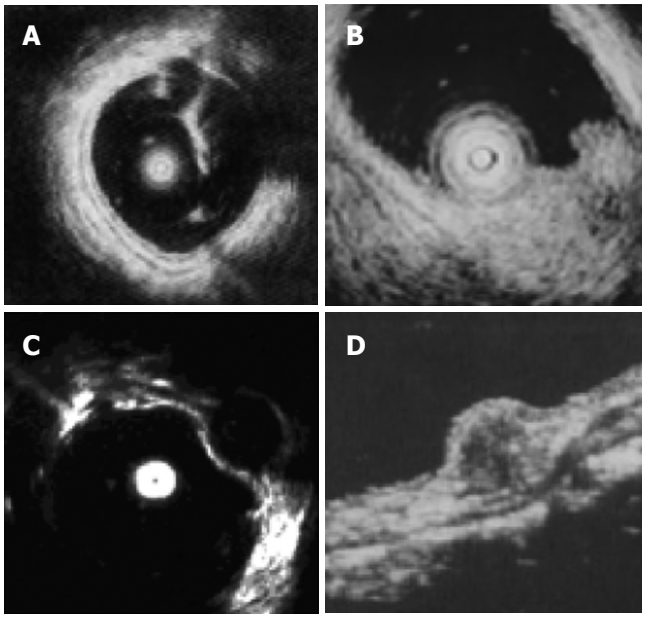

Figure 1 Endoscopic ultrasonography imagines of normal wall and submucosal tumors of the large intestine are presented. **A**: The normal wall displayed in 5 layers; **B**: Lipoma imagine showing a hyperechoic homogeneous mass located in the third layer; **C**: Leiomyoma imagine showing a hypoechoic homogeneous mass originated from the 4th layer; **D**: Rectal carcinoid imagine showing a submucosal hypoechoic mass with a homogenous echo. Courtesy by PH Zhou (Zhou, 2004 128 /id).

Three dimensional endoscopic ultrasonography

The need for three-dimensional (3D) EUS has arisen as a consequence of the difficulty less experienced endosonographers witness interpreting two-dimensional (2D) EUS images[34]. Indeed, 3D-EUS, compared to 2D-EUS, is relatively easy to use and the examination time will not be extended, as it is possible to view the whole lesion and perform a new scan immediately after a poor scan result[34].

However, some criterions are to be fulfilled in order to create a good image. The probe must be parallel and close to the mucosal surface. This is difficult in the stomach, but relatively easy in the esophagus, though probe wobbling can be caused by the peristalsis, respiratory movement

or cardiac impulse[34-36]. Furthermore, the time factor is critical, as the risk of probe wobbling increases with the time needed for completing a scan. In an investigation it took 3-4 s to complete a scan[34] as opposed to 3-5 min in another investigation[37]. More recent publications show promising results concerning the reduction of the time needed for processing the scans[35,36]. Finally, the size of the SMT is crucial. Due to the limited depth penetration in these probes, the results of 3D-EUS are better when applied to small SMTs, although the size of small SMTs (< 1 cm) tends to be overestimated by 3D-EUS[35,36].

3D-EUS data on GI SMTs are however sparse[36]. Thus, data on mucosal cancer is used in this paper to give an impression of 3D-EUS in practice. In a study of 43 upper GI lesions, depth staging was correct in 80% of the cases of esophageal cancer and almost 70% of the cases of gastric cancer, histologically confirmed. However, only 37% of the 3D-EUS images were of an acceptable quality, which meant that several images had to be made for each lesion[34].

Endoscopic ultrasound guided fine needle aspiration

Since it is impossible to differentiate definitely between benign and malignant SMTs by means of any imaging technique, histological or cytological confirmation is a necessity[16,30,38-40]. A study shows that only in 35% of cases was an acceptable submucosal representation achieved with forceps biopsy during standard endoscopy, even though the endoscopist intended to obtain submucosal tissue[2]. On the contrary, endosonographically performed fine needle aspiration (EUS-FNA) is a good method for obtaining cytological samples[30,39].

In EUS-FNA the aspiration needle can be inserted more precisely into the SMT than in percutaneous FNA[39]. Moreover, the incidence of malignant seeding is relatively low[15,39]. This may, however, be the result of selection bias: more biopsies are performed percutaneously and therefore more cases of cutaneous seeding than mucosal are seen. These advantages may be outweighed, though, by the risks of conscious sedation in endoscopy[39].

EUS-FNA contributes to solving therapeutic dilemmas. A study showed that due to the result of EUS-FNA the decision to abandon surgery was directly affected in 26% of patients with primary malignancies. The reason for this was severe malignancy, such as distant nodal metastasis[39].

The sensitivity of cytological samples achieved through EUS-FNA has been reported to be 88%-91% and the specificity close to a 100% for the diagnosis of malignant lesions confirmed by the surgical findings or long-term clinical follow-up[15,22,39,41]. However, as some investigators point out, in order to obtain an adequate cytological sample, the optimal situation is that a cytologist is present during the procedure[39]. Furthermore, there are different ways of handling the cytological samples obtained by EUS-FNA, such as performing smears and cell-blocks. It must be emphasized that neither mitotic counts nor immunohistochemistry can be performed on smears. Therefore the optimal situation is when cell blocks are made from the cytological sample. If the number of cells is too small to count mitotic figures per 50 high power fields, immunohistochemical staining with MIB-1 (a proliferation marker) can provide information of the cellular activity[30,42].

Sometimes examination of the whole SMT is needed in order to differentiate between benign and malignant, and a pitfall is the aspiration of normal smooth-muscle cells[22]. If possible, cells should be obtained from different parts of the SMT using a large needle (18-20G)[30].

Complications to EUS-FNA appear to be rare, as two investigations have shown a complication rate of 0%-2%[39,41]. However, careful Doppler-EUS examination must always be performed prior to EUS-FNA in order to prevent rupture of a possible varice[16].

Barium-contrast in X-ray

Barium studies can reveal several pathological conditions, such as submucosal infiltration, ulceration, mass presence and lumen stenosis, which may all be present in SMTs. Barium studies are also valuable in assessing the extent and multiplicity of SMTs[43]. The typical appearance of a SMT is an intramural mass with intact or ulcerated overlying mucosa[44]. The tumor is seen as a smoothly circumscribed mass, when seen en face and the margins as obtuse or right angles, when viewed in profile[45]. However, barium studies are limited to exophytic masses[44], and in staging and detection of early or subtle SMTs this method is of little value[43].

Computed tomography

Recent advances in CT have drawn attention to the use of CT for evaluating the GI tract[40]. Cross-sectional CT has the primary role in staging GI tumors[43]. New multi-slice CT has some advantages compared to single-slice spiral scanners, such as elimination of motion artifacts and acquisition of thinner sections. This improves the quality of 3D data, but the thin collimation involves an increase in the radiation dose to the patient[40]. CT has an advantage compared with EUS, namely the possibility of delineating the full extension of the tumor[44]. The forces of CT are demonstration of a tumor, its size, relation to adjacent organs and revelation of metastasis[46], and therefore are the tasks of CT primarily staging, surgical planning[47] and follow-up[47,48].

CT cannot classify SMTs as demonstrated in a recent study, where CT was inconclusive in more than 50% of GI stromal tumors (GISTs)[46]. CT cannot either differentiate between malignant and benign SMTs, unless obvious local invasion or metastases are present. CT, especially CT angiography, is valuable for the detection of gastric varices. In large and exophytic gastric stromal tumors, 3D-CT can be helpful in better characterizing the mass and determining its origin[40].

Traditional oral contrast agents of high attenuation have some disadvantages when evaluating the GI tract. An example is when the contrast does not mix uniformly with gastric contents, resulting in the creation of pseudotumors. Therefore water, which is of low attenuation, is preferred as an oral contrast agent. Simultaneously non-ionic contrast material is given intravenously, which enhances the GI walls. Furthermore, adequate distension of the stomach is important for proper imaging. Failure in the

latter may result in overlooked disease or the collapsed gastric wall mimicking disease[40].

Magnetic resonance imaging

Like CT, MRI is valuable in diagnosis and evaluating the extent of the tumor, including staging[49,50]. However, due to variable and non-specific appearances, MRI offers no additional information compared to CT concerning the internal features of, at least, GISTs[44].

Concerning limitations in both CT and MRI, it can be difficult to determine the organ of origin from cross sectional imaging alone in the presence of a significantly exophytic tumor[51]. However, MRI is a helpful adjunct to CT, especially concerning large SMTs, where the multiplanar capability of magnetic resonance can aid the determination of organ of origin, the relationship to other organs and delineate the major blood vessels. The new multi-channel CT-scanners have the same capability and may become the method of choice. In GISTs, the solid parts of the tumor are typically of low signal-intensity on T1-weighted images, but high signal-intensity on T2-weighted images. However, the degree of necrosis and hemorrhage greatly affect the signal-intensity pattern. Depending on the age of the hemorrhage, the signal-intensity will vary from high to low on both T1- and T2-weighted images. Due to gadolinium enhancement in viable tumor tissue, areas of necrosis can be outlined[45].

Positron emission tomography

In the recent years PET has shown to have great value primarily in the early assessment of response in GISTs to treatment with imatinib[48,52-54]. The reason for its effectiveness lies in the radiolabeled surrogate marker for glucose metabolism, fluorodeoxyglucose (FDG). FDG highlights areas of the body with enhanced metabolism, such as malignant SMTs[48]. Metabolic changes occur prior to morphological changes, which explains why several investigations conclude that PET is superior to CT and MRI in predicting early response to therapy[44,48,55]. A recent investigation on GISTs concludes that FDG-PET can separate imatinib-responders from -non-responders as early as 1 wk after initiation of treatment[54]. Furthermore, PET is indicated in cases, where equivocal CT- or MRI-images suspect metastases[47]. The risk of misinterpretation is minimized with the new combined PET/CT scanners uniting functional and morphologic imaging[27].

With PET, not only is the evaluation of response to therapy facilitated, but also the determination of the diagnosis, recurrence, staging and extent of disease[48,54,56].

To the disadvantages of PET count the fact that the acquisition time is 3-5 min per bed position. Due to respiratory motion, very small SMTs (< 5 mm) may be blurred and therefore missed[27].

As FDG is not a specific cancer tracer, uptake is seen in cicatrices following surgery due to benign inflammation, and therefore PET scans should not be performed until 3-4 wk after surgery to avoid these artifacts mimicking tumors. Other situations with increased uptake are tense muscles, catheters, tubes, stomas, the bone marrow in patients treated with chemotherapy and excretion of FDG to the urinary tract[57]. Physiological excretion of FDG can also be seen in the bowel, which can be difficult to differentiate from SMTs. Furthermore, it must be taken into consideration that slowly growing tumors, such as benign SMTs, only rarely absorb FDG. Moreover, hyperglycemia and administration of insulin may alter the distribution of FDG. Therefore at least 5 h of fasting and measurement of the blood glucose level prior to the scan is recommended[57].

BENIGN TUMORS

For all of the SMTs mentioned below, the endoscopic, EUS and macroscopic features can be seen in Table 1, and an EUS image of a leiomyoma and a lipoma is shown in the Figure 1.

Leiomyomas

Leiomyomas are the commonest mesenchymal tumors in the esophagus[58] as opposed to the rest of the GI tract, where GISTs are the most frequent[44]. Leiomyomas are found in the esophagus, colon and rectum, but are very rare in the stomach and small intestine[58].

Differential diagnoses to leiomyomas are preoperatively mostly leiomyosarcomas and, in the esophagus, carcinomas[59].

Schwannomas

GI Schwannomas are rare. Their ratio to GISTs, the most frequent GI SMTs, is approximately 1:50-100[58]. They are mostly found in the stomach.

Granular cell tumor

In the GI tract granular cell tumor mostly involves the middle to distal parts of the esophagus, with 1/3 of all the GI cases occurring at this site[4,60-63]. They are solitary in 80%-90% of all cases[62,63].

Heterotopic pancreatic tissue

Heterotopic pancreatic tissue is mostly located within 3-4 cm on both sides of the pylorus, but may occur in Meckel's diverticulum and rarely in the small intestine. Heterotopic pancreas is a nonneoplastic[22], congenital tumor thought to be a result of separation of fragments from the main pancreatic mass due to the rotation of the foregut[1,4,64]. An investigation found heterotopic pancreas in 0.25% of all explorative laparotomies[65].

A distinctive feature of heterotopic pancreatic tissue may be the presence of an opening, visible as a dimple on the surface[4,66], from which fluid may trickle on pressure[64]. Concerning differential diagnoses, both carcinoid tumors and heterotopic pancreatic tissue appear hypoechoic and irregular endosonographically[21].

Lipomas

GI lipomas occur throughout the GI tract, but are undoubtedly most frequent in the colon as a solitary, slowly growing, benign tumor, originating within the submucosa and protruding into the lumen[4,5,28].

CT findings are a well-circumscribed, submucosal lesion with uniform fat attenuation and, occasionally, a fibrous capsule[5]. X-ray criteria for lipomas are changing size and shape during the course of examination, reflecting their soft consistency[67].

Differential diagnoses from the endoscopic appearance are leiomyoma, neurofibroma, adenomatous polyp and villose adenoma. Differentiation is based on consistency, polypoid features and surface pattern of the different tumors[67].

Neurofibromatosis type 1

Solitary neurofibromas are rare. Therefore, neurofibromatosis should be suspected when neurofibromas are encountered[4]. Neurofibromatosis type 1 (NF1, von Recklinghausen Disease) is relatively common with a prevalence of 1/3000 births in Western countries[68]. The neurofibroma is derived from perineural cells on peripheral nerves[69]. GI involvement is common in NF1[28,50,70]. These SMTs have a predilection for the duodenum, especially the ampulla of Vater[71].

NF1 is associated with gliomas, meningeomas, phaeochromocytomas, hemangiomas and GISTs[28,50,72,73]. In the latter, the incidence of GISTs may be 200 times the incidence in an unaffected population[28,50,74]. The GISTs in patients with NF1 tend to be multiple[50]. Furthermore, it should be kept in mind that NF1 is also associated with carcinoid tumors that tend to occur at the ampulla of Vater, like the neurofibromas. The explanation for this co-location may be a transformation of an endo-ectodermal complex located near the ampulla of Vater in NF1-patients[75].

Vascular tumors

Hemangiomas: Multiple hemangiomas may be found, as in the blue rubber-bleb nevi syndrome that mostly affects the skin and GI tract[76]. Approaches to diagnosing vascular lesions are typically Doppler-EUS and CT-angiography[40], but a 99mTc-labeled redcell scan may also be performed to reveal hemangiomas or other transiently or mildly bleeding lesions[77], but endoscopy is regarded as the first choice. Logically, hemorrhage is a typical complication to hemangiomas[4]. One should keep in mind the differential diagnosis of esophageal and gastric varices[21].

Lymphangiomas: Lymphangiomas are rare, probably hamartomatous, anomalies that occur solitarily, mostly in the duodenum. Endoscopically, yellow-tan lesions are seen, occasionally with satellite lesions. When biopsy is performed, exudation of yellow chylous liquid is seen[4].

MALIGNANT TUMORS

Leiomyosarcomas

Leiomyosarcomas are mostly found in the small intestine[78], where they constitute more than 10% of all malignant lesions[79], and mostly behave in a highly malignant fashion[63]. A palpable abdominal mass may be encountered in almost 50% of cases of leiomyosarcomas in the small intestine[78].

Endoscopically, leiomyosarcomas are as a rule single and have a predominantly exophytic component[43,78].

Radiologically, excavated leiomyosarcomas may be confused with lymphomas and metastatic melanomas[43]. Leiomyosarcomas are expected to have a higher glucose metabolism than leiomyomas, and thus PET or PET/CT could aid the differentiation[54,80,81]. In addition, the EUS criteria mentioned in Table 1 may be helpful.

Gastrointestinal Kaposi's sarcoma

The causative viral agent of Kaposi's sarcoma is human herpes virus 8[4,28,82-84]. Kaposi's sarcoma is considerably more frequent in men than women and is mostly coursed by immunosuppression, especially HIV[63,84].

Endoscopically, Kaposi's sarcomas may be mucosal, but are usually submucosal and either isolated or extensively involve the bowel wall. All parts of the GI tract are at risk[4,63,85].

In the esophagus, acquired immunodeficiency syndrome-related lymphoma should be considered as a differential diagnosis, when viewed radiologically[43].

Metastases in the gastrointestinal tract

The most frequent primary tumors that result in GI metastases are breast cancer, melanoma and lung cancer[22]. The occurrence of metastases to the stomach from fatal breast cancer has been reported to be 8%[86]. Metastases may be brought about by hematogenic or lymphatic spread or seeding through the peritoneum[6,87].

Endoscopic findings are mainly sorted under three morphological features: nonulcerative SMTs, SMTs with elevation and ulceration at the apex (volcano lesions), and multiple nodules of varying sizes with tip ulceration[6]. EUS is valuable in evaluating the mode of spread, site of origin and the pathology[6].

Gastrointestinal stromal tumors

GISTs are the commonest mesenchymal tumors in the GI tract[88-90]. The annual incidence is estimated to be at least 10 to 20 cases per million[81,91]. Their origin is supposedly multipotential mesenchymal stem cells, and therefore both myogenic and neurogenic features may be present[1,46,92-96]. GI autonomic nerve tumors (GANTs), are now categorized under GIST owing to their great immunohistochemical and ultrastructural resemblance[97].

65% of GISTs occur in the stomach, 30%-35% in the small intestine and 5%-10% in the colon[98]. Colonic GISTs have a high proportion of malignancy[4,28].

Endosonographically, large size, lobulation, irregular borders and echogenic foci indicate malignancy (Tables 1 and 2)[42]. On CT, the signs that indicate a highly malignant tumor are calcification, ulceration, necrosis, cystic areas, fistula, metastasis, ascites and infiltration[46].

Endoscopic differential diagnoses are gastric lymphoma[99] and an inflammatory fibroid polyp[100]. There are quite a few differential diagnoses, when using CT, but if lymph node enlargement is seen, adenocarcinoma or lymphoma should be considered[44]. Differentiation is made with immunohistochemistry or electron microscopy.

CONCLUSION

Standard endoscopy, capsule endoscopy, push-and-pull enteroscopy, barium contrast X-ray and forceps biopsies can not differentiate between extraluminal compression and SMTs. Therefore, there is a need for EUS or whole

body imaging procedures as well as in the diagnosis of SMTs. EUS with biopsy is the first choice of diagnostic tool, but if depth penetration is improved in CP-EUS, this may be preferred due to the reduced number of intubations and examination time. So far 3D-EUS has not shown acceptable results, but it is expected to facilitate the assessment of borders, extent and size of SMTs in the future. Still, EUS is rather subjective and therefore the reproducibility of the results is reduced.

Biopsies should only be obtained, if the outcome could lead to a cancellation of a planned operation, due to the risk of malignant seeding in any malignant SMT[46] and due to the risk of hemorrhage if biopsies are taken from GISTs because of their brittleness[47].

Even in GISTs responsive to imatinib therapy, tumor size may decrease over months or not at all[47;48]. Therefore, with CT it may take months to reach conclusions regarding GIST responsiveness, whereas FDG-PET determines this within days to weeks after commenced treatment[48]. However, unless short-term follow-up is needed, CT is a sufficient way of monitoring. The quality of multi-slice CT is now comparable to MRI, but MRI has the advantage of disclosing necrosis, due to the enhancement of gadolinium in viable tumor tissue. MRI is especially an option when assessing liver metastases, while FDG-PET detects even small, malignant SMTs that may be overlooked by other diagnostic methods. Thus PET or PET/CT are recommendable for SMTs larger than 5 mm whereas (CP-) EUS is preferred for SMTs smaller than 5 mm.

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REVIEW

Classification of submucosal tumors in the gastrointestinal tract

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Abstract

This review is part two of three, which will present an update on the classification of gastrointestinal submucosal tumors. Part one treats of the diagnosis and part three of the therapeutic methods regarding gastrointestinal submucosal tumors. In the past there has been some confusion as to the classification of gastrointestinal submucosal tumors. Changes in classifications have emerged due to recent advances in mainly immunohistochemistry and electron microscopy. The aim of this paper is to update the reader on the current classification. Literature searches were performed to find information related to classification of gastrointestinal submucosal tumors. Based on these searches the twelve most frequent submucosal tumor types were chosen for description of their classification. The factors that indicate whether tumors are benign or malignant are mainly size and number of mitotic counts. Gastrointestinal stromal tumors are defined mainly by their CD117 positivity. In the future, there should be no more confusion between gastrointestinal stromal tumors and other types of submucosal tumors.

Key words: Submucosal tumor; Immunohistochemistry; Smooth muscle derived submucosal tumors; Submucosal tumors of neurogenic origin; Gastrointestinal stromal tumor; Malignant; Benign

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INTRODUCTION

Submucosal tumors (SMTs) are mesenchymal tumors and as such, they may have very diverse origins. SMTs were originally divided into being of muscular or neural derivation. However, in the past decade it has become more obvious that the SMT group, gastrointestinal stromal tumors (GISTs), cannot be placed in any of these groups. This conclusion has been drawn based on the electron microscopic and immunohistochemical features, since GISTs in about 95% of cases stain positively for the protein CD117[1-3]. This protein is not expressed by any of the other SMTs, except for heterotopic pancreatic tissue[3], which however does not pose a differential diagnosis since it is easily differentiated from GISTs by light microscopy. However, as metastases from various sites may also present as SMTs, there is almost no limitation to the origin of SMTs.

Differing between benign and malignant SMTs may course few problems, whereas it creates a special obstacle to distinguish between a benign SMT and the potentially malignant GIST. GISTs may appear benign both in mitotic counts and lack of cellular atypia, but still behave malignantly[4]. Therefore it is of great value that immunohistochemistry has been introduced, since staining for few proteins provides the information for this classification.

The implementation of immunohistochemistry in the definition of GISTs and other recent chances regarding the classification of SMTs, have created a need for a review in this field. The aim of this paper is to update the reader on particularly immunohistochemistry, morphology, and other characteristics of the 12 most frequently encountered SMTs for classification purposes, grouped as benign or malignant.

BENIGN SUBMUCOSAL TUMORS

SMTs smaller than 3 cm are generally considered benign tumors. The number of mitotic counts allowed for benign SMTs varies among the different SMTs.

Leiomyomas

On cut section leiomyomas have a pale, firm, rubbery or whorled appearance[6]. Microscopically, they constitute of a bland spindle cell population arranged in fascicles and whorls. Mitoses are lacking or few in number and necrosis is normally absent[7]. Concerning immunohistochemistry,

leiomyomas are globally positive for desmin and smooth muscle actin, but negative for CD34 and CD117 proteins[8].

Malignant change is very rare in leiomyomas. Thus, a leiomyoma does not represent a presage of a leiomyosarcoma in most cases[10-12].

Schwannomas

On cut section, Schwannomas are grey in color[13]. Microscopically, spindle cells are seen with vague nuclear palisading. There are often sprinkled lymphocytes and a nodular lymphoid cuff[8,9,13]. Immunohistochemically, Schwannomas are positive for S100-protein and vimentin[3,9,13].

Schwannomas are always benign and have never been reported to develop malignancy[8,9]. Therefore, it is important to differentiate them from GISTs, which they resemble both grossly and clinically. Immunohistochemistry provides the sufficient distinction[9,13].

Granular cell tumors

This benign neoplasm is of neural origin (Schwann cell) and often involves peripheral nerves in mucosa or connective tissue[14,15].

Microscopically, granular cell tumors typically infiltrate between adjacent tissues and the overlying mucosa may show pseudocarcinomatous hyperplasia[15,16]. Furthermore, they most commonly appear as sheets of uniform histiocyte-like cells with an abundant, eosinophilic, periodic acid-Schiff-reaction-positive cytoplasm containing lysosomal granules and small vesicular nuclei[7]. Immunohistochemically, granular cell tumors are S100 protein- and neuronspecific enolase-positive, lending support to their neural derivation[1,7,9,16].

Malignant change is very rare and based strictly on the presence of metastases[7].

The firm consistency of granular cell tumors makes it difficult to achieve a biopsy[14]. If the biopsy is too superficial, granular cell tumors may be confused with squamous cell carcinoma, since the overlying mucosa may show pseudocarcinomatous hyperplasia[16].

Heterotopic pancreatic tissue

The cut surface of heterotopic pancreatic tissue is typically tan[17]. If the covering mucosa is intact, the heterotopia appears smooth-walled and well circumscribed[18]. Microscopically and immunohistochemically, heterotopic pancreas may contain all features of a normal pancreas[1,18,19].

Though rare, malignancy in heterotopic pancreas must be considered[1,19-21].

Differential diagnoses. If mucus retention is present, heterotopic pancreatic tissue can be hard to differ from duplication of the stomach and mucinous carcinoma[19]. If acini and ducts are missing, it may be misinterpreted as an adenomyoma[17].

Lipomas

The cut surface in a lipoma is homogenously yellow, lobulated and has the appearance of adipose tissue[17,22]. Microscopically, lipomas are composed of mature adipose tissue surrounded by a fibrotic capsule[1,9]. They arise mostly from submucosal fat, but infrequently from subserosal fat[22,23]. Fat cells are S100 positive, and CD34 positive spindle cells may be seen, but immunohistochemistry plays little role in the diagnosis of lipomas[24].

Neither solitary lipomas nor lipomatosis has a malignant potential. Liposarcomas are exceptionally rare and will therefore not be mentioned further in this review[1].

Neurofibromatosis

Neurofibromas are classified into three groups as either being localized, diffuse or plexiform, the latter being pathognomonic for neurofibromatosis type 1 (von Recklinghausen Disease). Diffuse neurofibromas are rare in the GI tract[25,26]. They normally involve the myenteric nerve plexus[25].

Macroscopically, localized neurofibromas are fusiform or diffuse tumors with a gray or tan cut surface. Plexiform neurofibromas have a ropelike appearance, when they involve non-branching nerves, but are described as "a bag of worms", when they involve highly branching nerves[25].

Localized and plexiform neurofibromas have the same microscopic appearance, but the latter is organized into multiple fascicular units. The tumors consist of spindle cells loosely arranged (Schwann cells and fibroblasts) with varying amounts of intervening collagen. Frequently, accumulation of mucopolysaccharides results in a gelatinous or myxoid tumor[25,27]. Immunostaining for S-100 may reveal residual myelinated nerve fibers[25].

Malignant progression may be seen especially in patients with plexiform neurofibromas forming malignant peripheral nerve sheath tumors[28].

Vascular tumors

Hemangiomas: Hemangiomas are classified into three major types: capillary, cavernous or mixed. The former is the commonest and results in small tumors, contrary to the cavernous, which may involve long segments and all wall layers of the ileum[1]. Hemangiomas represent either true neoplasms or hamartomas[29]. Microscopically, sheets of spindle cells are seen, interspersed by clusters of erythrocytes[9]. Immunohistochemically, hemangiomas are positive for CD31, CD34 and factor Ⅷ[3,9].

Lymphangiomas: Histologically, the presence of lymphocytes in lymphangiomas aids the differentiation from hemangiomas[1]. Immunohistochemically, lymphangiomas are typically factor Ⅷ and D2-40 positive, where D2-40 is more specific and aids the differentiation from hemangiomas[3].

MALIGNANT SUBMUCOSAL TUMORS

SMTs larger than 3-5 cm, with mitotic counts greater than 2 per 10 high power fields or that involve more layers are generally considered high-risk tumors for malignancy. GISTs have another classification, as described below[2,9].

Leiomyosarcoma

Leiomyosarcomas are predominantly exophytic and macroscopically visible (Figures 1 and 2)[30,31]. Microscopically,

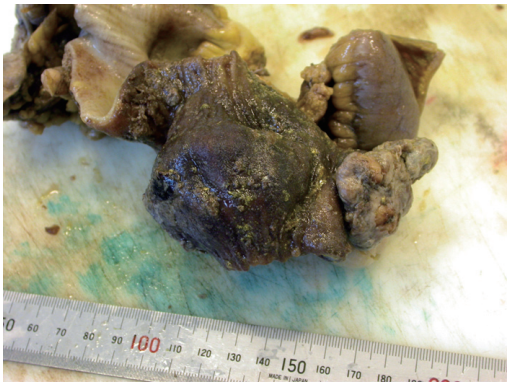

Figure 1 Exophytic leiomyosarcoma of the ileum, measuring 6 cm x 5 cm x 3 cm. A fibrin coated mucosa with stigmata of hemorrhage and discolorations of the adjacent mucosa can be seen (Courtesy by S Duun).

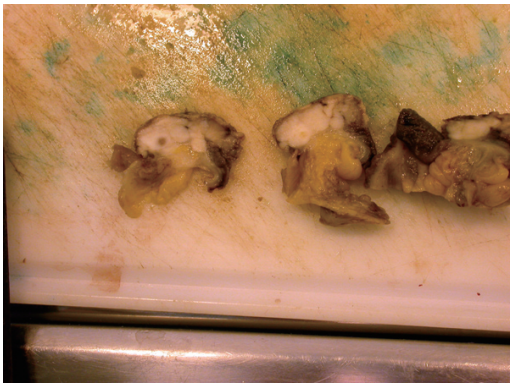

Figure 2 Cut surface of the leiomyosarcoma presented in Figure 1, showing a possible necrosis and a white, fish-flesh-like color, as typical for sarcomas. The surface did not bulge on incision (Courtesy by S Duun).

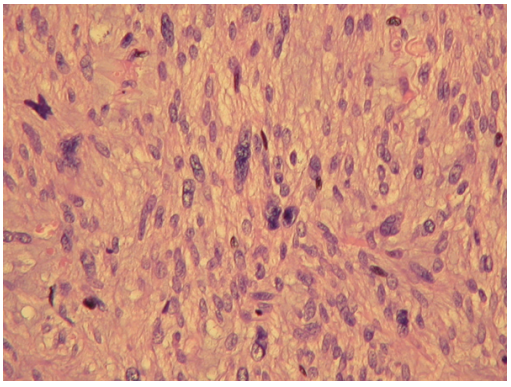

Figure 3 The leiomyosarcoma presented in Figures 1 and 2. It is of low malignancy, but shows nuclear atypia, pleomorphism and mitoses. (HE, x 100) (Courtesy by S Duun).

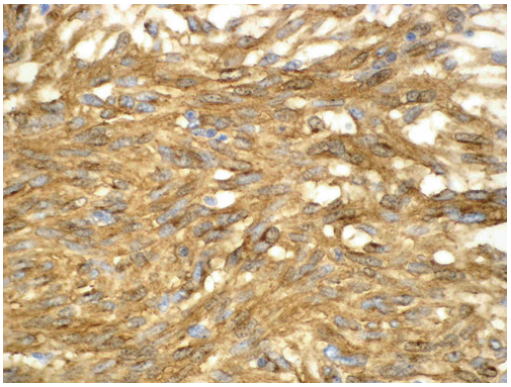

Figure 4 Histological findings of a GIST showing a positive CD117 immunohistochemical reaction. (× 200) (Courtesy by B Vainer).

necrosis, cellular and nuclear pleomorphism, mitotic figures and atypical mitoses are typically seen (Figure 3)[17,30,32]. There may be areas of fibrosis, hyalinization or necrosis[17]. Leiomyosarcomas are positive for desmin and smooth muscle actin, but negative for CD34 and CD117[9].

Differentiation between leiomyosarcomas and leiomyomas is difficult, but leiomyosarcomas may possess typically malignant features as disorganized microscopic appearance, a high mitotic index and the presence of metastasis[31].

Gastrointestinal Kaposi's sarcoma

Microscopically, Kaposi's sarcoma exhibit erythrocytes trapped in clefts in pleomorphic spindle cells, and may therefore be classified as a vascular tumor[1,9]. Kaposi's sarcomas are positive for vimentin and smooth muscle actin and typically also for CD31 and CD34. A little more than 50% are positive for factor Ⅷ[3]. Furthermore, human herpes virus 8 can be demonstrated by polymerase chain reaction[9,33].

The most important differential diagnosis is bacillary angiomatosis[34]. Additionally, flat Kaposi's sarcomas may be confused with a cytomegalovirus (CMV) lesion[7].

Metastases in the gastrointestinal tract

Microscopic and immunohistochemical similarity between the primary tumor and another tumor suggests metastasis[35,36]. Immunohistochemistry may therefore be essential in determining the origin of the metastasis[36]. Differential diagnoses to metastases are mainly primary tumors[35,37].

Gastrointestinal stromal tumors

Immunohistochemistry was the reason for the introduction of the GIST appellation in 1983[38]. Still, many SMTs were misclassified as GISTs and vise versa until recently[5,39].

Macroscopically, low-risk GISTs are typically circumscribed but not encapsulated. The cut surface is without whorls and has a characteristic grey color. High-risk GISTs are sarcomatous on the cut surface, white, fish flesh like, and may show signs of hemorrhage, calcification, ulceration, necrosis, cystic areas and myxoid degeneration. However, these features may also be seen in larger low-risk GISTs. Neither endophytic features nor ulcerations necessarily equals malignancy[1,4,40].

Microscopically, GISTs typically have spindle cell morphology, but epithelioid morphology may be seen[1,8,41]. Immunohistochemically, CD117 protein is a rather specific marker for GISTs with 95% positivity for the protein (Figure 4)[1-3]. The World Health Organization suggests that this may be the single, best defining feature of GISTs. The 5% of GISTs negative for CD117 are due to artifacts, sampling errors, clonal evolution (perhaps in imatinib

treatment) and only 2% actually lack CD117[3,5]. The latter seem to have mutations in platelet derived growth factor receptor alpha (a CD117-related tyrosine kinase receptor) instead[42]. About 70% of GISTs are positive for CD34[3]. Furthermore, nearly all GISTs will show diffuse and strong staining for vimentin[3]. A recent investigation has shown no significant correlation between survival, histological tumor type (epithelioid or spindle cell) and CD34 immunoreactivity (positive versus negative)[43]. Electron microscopic features are a mixture of autonomic nerve and smooth muscle cells[9,44].

About 20%-30% of all GISTs display malignant behavior[45,46]. All GISTs are potentially malignant and thus cannot be classified as benign versus malignant. Instead, they are regarded as being of very low risk (tumor < 2 cm and < 5 mitoses/50 high power fields), low risk, intermediate risk or high risk (tumor > 5 cm and > 5 mitoses/50 high power fields or tumor >10 cm regardless of mitotic activity) for recurrence and metastasis or overtly malignant (proven metastases at initial diagnosis)[1,43,47]. A recent study has found a perhaps more clinically useful classification focusing on three factors: tumor size (smaller or larger than 5 cm), hemorrhage/necrosis (absence or presence) and Ki-67 LI (proliferation marker; more or less than 3%), which shows significant difference between benign and malignant defined this way[45].

As typical for sarcomas, GISTs generally do not metastasize to the regional lymph nodes[48], but instead spread hematogenously to the liver or metastasize to the peritoneum[41,49,50]. These are also the commonest sites of recurrence[47,51]. A few GISTs seem to lack mitotic activity, but still metastasize[4,5]. Due to this unpredictable behavior, all GISTs must be treated as potentially malignant.

Microscopic differential diagnoses are leiomyoma, leiomyosarcoma, Schwannoma (if the nuclei have a palisade conformation), neurofibroma and more[48]. Differentiation is made with immunohistochemistry or electron microscopy.

CONCLUSION

The combination of size, histological, immunohistochemical and, if possible, ultrastructural criteria is the most precise way of classifying SMTs and defining benign or malignant properties. Concerning the possibility of malignancy, this should always be considered when SMTs are larger than 3 cm or with mitotic counts greater than 2 per 10 high power fields. However, GISTs should always be considered potentially malignant.

Smooth muscle derived SMTs (e.g. leiomyomas and leiomyosarcomas) stain strongly and diffusely for desmin and smooth muscle actin and are negative for CD34 and CD117 as opposed to GISTs, which are mostly positive for the CD34 and CD117 biomarkers, with the latter being an almost specific marker for GISTs. Accordingly mesenchymal tumors are now less likely to be misclassified.

As a third group, SMTs of neurogenic origin (e.g. Schwannomas, granular cell tumors and neurofibromas) typically show positivity for S100 and negativity for desmin, actin and CD117. Vascular tumors (e.g. hemangiomas, lymphangiomas and Kaposi's sarcomas) are typically factor Ⅷ positive. Immunohistochemistry plays little role in the diagnosing of lipomas and heterotopic pancreatic tissue, as their microscopic appearance is easily recognized.

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REVIEW

Therapeutic procedures for submucosal tumors in the gastrointestinal tract

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Abstract

This review is part three of three and will present an update on the therapeutic options and procedures concerning gastrointestinal (GI) submucosal tumors (SMTs). The aim of this paper is to investigate the treatments of GI SMTs and to present a case of a gastrointestinal stromal tumor (GIST). Literature searches were performed to find information on therapy for GI SMTs. Based on these searches, the optimal therapeutic procedures could be outlined. The choice of treatment of localized tumors is endoscopic resection if possible or, alternatively, laparoscopic resection or surgical resection by an open procedure. However, benign SMTs should only be excised if symptoms are present, and GISTs should be treated with particular precautions. Irresectable or recurrent GISTs may be successfully treated with the tyrosine kinase inhibitor, imatinib.

Key words: Submucosal tumor; Treatment; Case story; Endoscopic mucosal resection; Imatinib

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INTRODUCTION

Surgical resection is the golden standard for treatment of gastrointestinal (GI) submucosal tumors (SMTs). However, new surgical and medical therapeutic options for SMTs have emerged recently. These include, primarily, endoscopic resection and usage of the tyrosine kinase inhibitor, imatinib.

The choice of treatment is based on whether the SMT is thought to be: benign, malignant, exophytic, endophytic, its size, extent and the presence of symptoms. Treatment is mostly not indicated in an asymptomatic, benign SMT, found incidentally. These SMTs are instead controlled by follow-up examinations[1]. On the contrary, malignant SMTs should be excised surgically as a rule[2,3].

Gastrointestinal stromal tumors (GISTs), the most common mesenchymal tumors in the gastrointestinal tract, form a specific problem as they have the ability to metastasize, even though they may appear to be fully benign[4,5]. In recent years it has become clear that the tyrosine kinase inhibitor, imatinib, has a place in the treatment of inoperable, recurrent and metastatic GISTs[2,6-8].

Recent validation of these new treatment options has created a need for a review of the therapeutic options when dealing with SMTs. The aim of the present paper is to update the reader on the different therapeutic possibilities, mostly surgical, regarding various types of SMTs. A case story of a GIST is presented in this context.

ENDOSCOPIC SURGERY

For SMT resection, endoscopic surgical procedures represent an alternative to laparoscopic and conventional open surgical bowel resection procedures in selective cases. Endoscopic ultrasonography (EUS) and multi-slice CT are helpful tools for deciding on which type of surgical procedure should be performed[1]. A comparison between the different methods can be viewed in Table 1.

Standard snare polypectomy is performed with either a one- or a two-channel endoscope. With the one-channel endoscope, the cauterizing snare is placed around the SMT base and pulled as the resection is performed. With the two-channel endoscope, the oral part of the SMT is grasped with a forceps and held, while the snare is placed around the SMT[1].

Strip biopsy is initiated with a submucosal injection of physiologic saline, which may be EUS-guided. This separates the muscularis propria from the luminal layers. After placing a snare around the SMT base, excision is done with electrocoagulation while tightening the snare[1,9,10].

Resection can also be performed with a ligation

Table 1 Various endoscopic therapeutic procedures for the treatment of SMTs

	Indication	Contraindications[1]	Complications	Advantages	Disadvantages
SSP[1,56]	SMT < 2 cm; polypoid/ pedunculated; sessile with a base < 1-2 cm; intraluminal and originating in muscularis mucosa or submucosa	SMT > 2 cm; originating from the muscularis propria; intramural SMT; extraluminal SMT; located on the lesser curvature, posterior aspect of the stomach body or the cardia	Incomplete resection, hemorrhage, perforation (when the SMT is > 2.5 cm)	High success rate, few complications	See "Complications"
SB[1,9,10,57,58]	Same as for SSP	Same as for SSP	Minor bleeding treated with saline inj., metal clips or liquid thrombin	The saline injection prevents full-thickness burning and perforation; high success rate; safe, quick and easy method	If the saline is injected in the surrounding tissue, the SMT will become sessile and therefore more difficult to remove
ESMR-L[1]	SMT < 1 cm	SMT > 1 cm; originating from the muscularis propria	No serious complications have been reported	Not restricted by the location of the SMT; achieves deeper resection than SB and conventional EMR and thus a higher rate of curative resection	This technique can only be applied to small SMTs
ESMR-C[1]	SMT < 2 cm	SMT > 2 cm; SMTs in the muscularis propria	Minor hemorrhage, though rare.	Simpler and easier version of EMR; high success rate; saline inj., see SB	See "Complications"
UT[1,58]	Simple and multicystic SMTs (e.g. lipomas and cystic lymphangiomas)	Vascular tumors	Hemorrhage	Reduced risk of perforation, due to the fact that only the upper half is removed; can be applied to larger tumors	Only applicable in cases of lipomas and cystic lymphangiomas
EE-M[1]	Easiest if well capsulated; large SMTs and SMTs in the muscularis propria can be removed by this technique	SMTs with wide bases, severe adhesions or not well capsulated	Minor hemorrhage	Can be used to resect leiomyomas originating from the muscularis propria; sessile or large SMTs > 2 cm can be resected	Very difficult to perform
EE-I[1]	Large SMTs and SMTs in the muscularis propria can be removed by this technique	Unknown since this is a new technique	Perforation, minor hemorrhage	Like EE-M this technique is not limited by the size, sessile form or association with the muscularis propria	New method, which means that the efficacy and safety is not known for sure

[1]These are not absolute contraindications, but should rather be seen as circumstances, where resection is complicated. SSP: standard snare polypectomy; SB: strip biopsy; ESMR-L: resection performed with a ligation device; ESMR-C: endoscopic submucosal tumor resection with a transparent cap; UT: unroofing technique; EE-M: endoscopic enucleation performed with an initial mucosectomy; EE-I: endoscopic enucleation performed with an insulated-tip electrosurgical knife.

device. After a submucosal saline injection the SMT is aspirated into the ligation device and the elastic band is released around it. Snare resection is performed with electrocoagulation below the elastic band. Endoscopic SMT resection with a transparent cap is a simpler and easier method[1].

With the unroofing technique, the upper half of the SMT is resected with a snare, creating an opening in the overlying mucosa. In most cases, the remnant SMT resolves spontaneously[1].

Endoscopic enucleation can be performed with an initial mucosectomy. The superficial part of the tumor is removed employing a snare or a cutting knife. Then a biopsy forceps is used to separate the SMT from the surrounding tissue, and the tumor can be removed with a snare[1].

Endoscopic enucleation can also be performed with an insulated-tip electrosurgical knife. Epinephrine injected in the proximal aspect of the SMT detaches it from the overlying tissue. Using a needle-knife, a 3-5 mm diameter hole is made. With the insulated-tip electrosurgical knife introduced though the hole, a longitudinal incision is made in the overlying mucosa and the surrounding tissue is dissected away. The tumor can now be removed en bloc[1].

Some therapeutic interventions can also be performed with push-and-pull enteroscopy in selected cases, which is typically a symptomatic, benign, small intestinal SMT[11].

TREATMENT OF BENIGN SUBMUCOSAL TUMORS

Benign SMTs should generally only be treated if they are symptomatic. In case of asymptomatic SMTs, follow-up examinations seems to be the best approach[1]. Exceptions from this rule (e.g. heterotopic pancreatic tissue) will be dealt with in the following.

Leimyomas

Small, symptomatic, duodenal leiomyomas with benign features can be safely treated with local excision *via* a longitudinal duodenotomy[12]. If the leiomyoma is located in the esophagus it will often result in progressive dysphagia, in which case enucleation or resection is required[13]. Endoscopic excision is also an option, and even leiomyomas larger than 2cm can be removed by enucleation using a snare, cutting knife or an insulated-tip electrosurgical knife, see above[1]. A case report has shown a successful resection of an esophageal leiomyoma by

means of thoracoscopic enucleation[13]. In asymptomatic leiomyomas, follow-up may be preferred[1].

Schwannomas

Since GI Schwannomas are always benign, removal is only indicated in case of severe symptoms[14].

Granular cell tumors

In case of symptoms, endoscopic tumor excision is a good alternative, when the tumor is restricted to the inner layers, as recurrence or metastasis has never been documented in any patients[15,16]. When the tumor also invades the outer layers, EUS can contribute to planning the surgical resection[15].

An investigation of laser therapy for esophageal granular cell tumors included four patients. The method was successful in achieving complete necrosis of the esophageal changes, necessitating four sessions per patient. A mean follow-up period of 66 mo showed no evidence of tumor recurrence. No complications were observed leaving laser therapy as a putative new therapy in selected cases[17].

Heterotopic pancreatic tissue

Malignancy in heterotopic pancreas must be considered, although it is relatively rare[18-21]. If symptoms occur, surgery may be a necessity[18]. Endoscopic resection may be performed either by standard snare polypectomy, strip biopsy, resection performed with a ligation device or by endoscopic submucosal tumor resection with a transparent cap[1]. If heterotopic pancreatic tissue is found incidentally during operation for other reasons, prophylactic resection of the tissue is advisable for prevention of later complications[22].

Lipomas

Large lipomas may cause massive bleeding or intussusception[1]. If symptoms occur, the treatment of choice is surgical removal[23-26]. If the tumor is small, endoscopic polypectomy or enucleation may be preferred[23]. Large lipomas can be removed with the unroofing technique[1]. Asymptomatic lipomas should be followed without surgery[24,26] and some of them may in fact resolve spontaneously[1].

Neurofibromas

Neurofibromas are not easy to treat, as they may seem well defined macroscopically, but microscopy often reveals local infiltration. Therefore, these tumors commonly recur after excision[27]. Accordingly surgical resection has to be recommended due to high frequency of recurrence.

VASCULAR TUMORS

Hemangiomas

The therapeutic strategy depends on the size, number, location and symptoms[28]. Endoscopic coagulation or removal of a recurrently bleeding hemangioma may be performed either as an exploratory laparotomy with excision of the hemangioma or as laparoscopic excision with preceding push-and-pull enteroscopy, where the hemangioma is marked with ink[29]. However, in blue rubber-bleb nevi syndrome where multiple hemangiomas may be present, complete eradication may be impossible[28]. Alternatively, endoscopic laser photocoagulation or plasma argon coagulation may be performed[28].

Lymphangiomas

Large, symptomatic lymphangiomas can be removed endoscopically with the unroofing technique[1].

TREATMENT OF MALIGNANT SUBMUCOSAL TUMORS

Leiomyosarcomas

As leiomyosarcomas are considered radio- and chemoresistant[30], surgical resection remains the only effective treatment and involves both the tumor and adjacent mesentery in small-intestinal leiomyosarcomas[3].

Kaposi's sarcoma

Kaposi's sarcoma typically occurs in the coexistence of human herpes virus 8 and HIV[31,32]. The classical Kaposi's sarcoma is rarely fatal contrary to the much more frequent HIV-associated variant[31]. The treatment is usually dictated by the presence of symptoms[33], and should initially include highly active antiretroviral therapy against HIV with or without specific anti-Kaposi's sarcoma therapy. This has been shown to halt progression or induce regression. Kaposi's sarcoma is moderately responsive to radiation and chemotherapy[32].

Metastases

Treatment of metastases is angiographic embolization to control active tumor bleeding, endoscopic removal of the metastases, surgical exploration or medical treatment. If multiple organ involvement is present, and there is no active bleeding, the indication for treatment is questionable[34].

Treatment of gastrointestinal stromal tumors

GISTs stand out as especially complicated to treat compared to other SMTs. Therefore the treatment of these tumors will be described in more detail.

Surgical approaches: laparotomy or laparoscopy?

The first choice of treatment of localized GISTs is complete surgical resection, which seems to be the most important prognostic criterion[2,8,35,36]. The tumor should be removed en-bloc respecting a possible pseudocapsule to avoid intraperitoneal dissemination[6,36-38], and therefore adjacent organs adherent to the GIST should be resected en-bloc with the tumor[2,36]. GISTs should be resected aggressively with a tumor-free margin[2,6,37,39], and determining this is mostly not much of a problem, since GISTs tend to be exophytic[36]. Re-excision should be considered in case of intramural GISTs that have been excised intra-lesionally and do not infiltrate the serosal surface[2]. A consensus meeting in 2005 concluded that

laparoscopic surgery should be avoided, especially in GISTs larger than 2 cm, due to the risk of rupture[2]. Yet recent studies of even very large (up to 15 cm)GISTs showed successful and safe resection in nearly all of the patients employing laparoscopic resection. The reason for unsuccessful laparoscopic treatment (1 patient out of 64) was conversion to laparotomy due to suspected bowel injury when establishing pneumoperitoneum[37,40]. Lymphadenectomy is not a routine procedure owing to the route of malignant spread in GISTs, which is mainly hematological metastasis to the liver[2,6,39]. Hepatectomy for liver metastasis is not recommended as it does not seem to increase survival rates[41]. If the GIST is large or involves large vessels embolization should be considered.

Tyrosine kinase inhibitors-imatinib

GISTs are chemo- and radioresistant[42,43]. Immediate medical treatment with the tyrosine kinase inhibitor, imatinib, is indicated in case of metastatic, recurrent or irresectable GISTs[2,6-8]. Imatinib should also be considered in case of equivocal images[2]. The effect can be monitored with combined positron emission tomography (PET) and CT, PET-CT[44]. Imatinib is given as an oral treatment, with a recommended daily dose of 400 mg[2,7]. For lack of response, 600-800 mg/d may be attempted[2]. Treatment with imatinib should be continued until progression, intolerance or patient refusal[2].

Imatinib specifically inhibits a mutated tyrosine kinase receptor (kit-receptor; CD117) that normally regulates cell growth and survival, but a gain-of-function mutation has made it continuously active. However, imatinib has also shown to inhibit platelet derived growth factor receptor alpha mutations (a CD117-related tyrosine kinase receptor) and tumors without mutations[2,7,8,44-47] (e.g. neurofibromatosis type 1-associated GISTs)[48]. A reason for the dramatic effect of imatinib is probably that it inhibits the kit-receptor signaling, which secondarily inhibits the glucose uptake and metabolism and thus cell proliferation[44,49].

The effect of imatinib on GISTs often results in increased tumor size due to hemorrhage, edema and myxoid degeneration and therefore do not correlate to the response criteria of the World Health Organization or of Response Evaluation Criteria in Solid Tumors[2,50]. Decreased metabolism in fluorodeoxyglucose marked PET (FDG-PET), reduction in tumor density (Hounsfield units) in CT and symptomatic improvement all indicate tumor response to imatinib[2]. Long-term studies are still not available. However, patients did not survive for more than 1 year earlier, but with imatinib therapy they now live for more years[51]. High and intermediate risk GISTs should be followed with a CT scan every 3-4 mo for 3 years, then every 6 mo until 5 years and yearly thereafter. Low and very low risk GISTs can be followed every 6 mo for 5 years[2].

The side effects from imatinib tend to be mild and occur rather infrequently[44,50,52]. However, lethal complications such as bleeding may occur[50].

CASE STORY

A 61-year-old woman was hospitalized due to black stools for three days, fainting fits and hematemesis during the past week. The patient suffered from mild epigastric pain and had also experienced nausea.

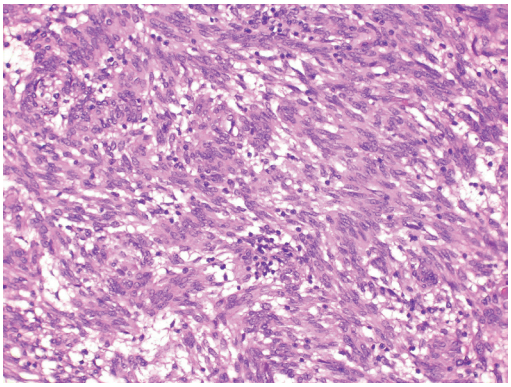

Figure 1 Histological findings of the gastrointestinal stromal tumor described in the case are presented showing spindle shaped cells with mild, nuclear atypia, few mitoses and slight, diffuse lymphocyte infiltration is seen (HE, x 100). Courtesy of B. Vainer.

On examination, the patient was found to be slightly tender corresponding to the epigastrium. Black feces were found at rectal exploration and fresh blood appeared from the stomach tube. Hemoglobin was only 4.5 mmol/L (reference interval: 7-10) at admission.

At standard upper endoscopy a 4 cm × 4 cm SMT was revealed in the anterior wall of the stomach, close to the cardia. It had a fibrin-coated ulceration showing stigmata of hemorrhage. The biopsies were inconclusive, due to lack of submucosal representation.

A CT scan confirmed the gastric mass, but also revealed a mass in the left adrenal gland. It was not possible to take a biopsy from the latter by ultrasound due to lack of visualization. Neither was it possible by CT owing to the fact that there was no free window to reach the tumor without serious risk of lung damage.

The patient was referred for resection of both tumors by an open surgical procedure. Postoperatively, an explorative laparotomy was performed due to non-specific hemorrhage. Furthermore, bilateral, moderate pleura exudates were found, however not requiring drainage. Apart from this, the postoperative course was uneventful.

Macroscopic examination showed a tumor size of 45 mm × 40 mm × 36 mm with a cystic lumen of 37 mm containing blood and mucus. The consistency of the tumor tissue was firm, it had a capsule-like structure with fibrous septa and the color was mixed gray-yellow and brown. Microscopically, the tumor tissue was whirled with distinct palisading nuclei (Figure 1). The cells were spindle shaped with mild nuclear atypia and few mitoses (1-2/50 high power fields). There was significant edema and mild, diffuse lymphocyte infiltration. Furthermore, central degeneration with sequelae from hemorrhage, fibrosis and coagulation necrosis surrounding vascular structures was seen. Additionally, multiple small, thin-walled cysts looking like dilated lymph vessels and invaginated serosal surface was found. No tumor necrosis was seen.

Immunohistochemically, the tumor tissue was strongly reactive for CD117 and CD34 (Figures 2 and 3) with smaller areas being positive for smooth muscle actin, and

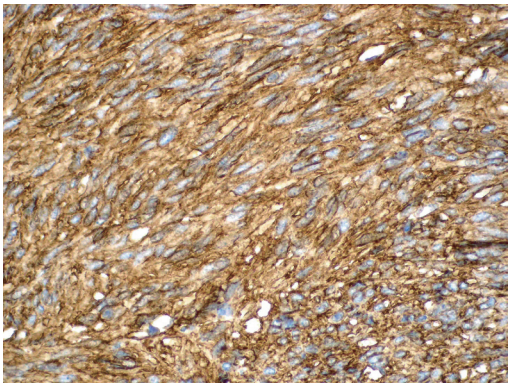

Figure 2 Histological findings of the gastrointestinal tumor described in the case are presented showing a positive CD34 immunoreaction (x 100). Courtesy of B. Vainer.

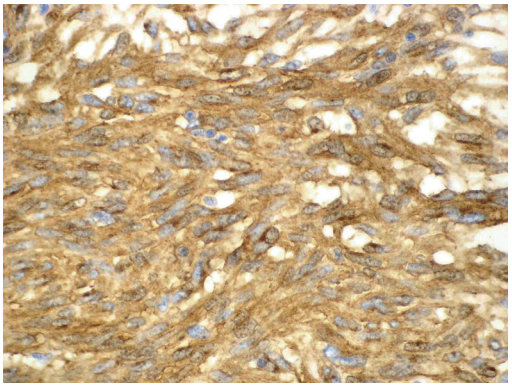

Figure 3 Histological findings of the gastrointestinal stromal tumor described in the case are presented showing a positive CD117 immunoreaction (x 200). Courtesy of B. Vainer.

negative for desmin, S-100 and cytokeratin. MIB-1 (Ki-67, proliferation marker) was reactive in 1%-2% of the cells.

In conclusion, the tumor was found to be a low-risk GIST with microscopically confirmed free resection margins. The adrenal tumor was a cortical adenoma and was not related to the GIST.

DISCUSSION

The choice of surgical procedure is dictated by the clinical condition of the patient, the type, size, shape, location and extent of the GI SMT. In general, benign appearing, asymptomatic SMTs should be evaluated by follow-up examinations, whereas surgical resection should be reserved for symptomatic SMTs or those suspicious of malignancy, including all GISTs[53,54].

The surgical procedure can either be performed endoscopically for intraluminal growing SMTs, laparoscopically for SMTs with extraluminal growth or through laparotomy for SMTs suspected to be malignant[39,55]. Employing endoscopic resection, there is an increased risk of perforation and hemorrhage, if the SMT is located near the serosa, but this may be prevented by the application of metal clips[1]. The availability for expertise in endoscopic and laparoscopic procedures will be a limiting factor until these techniques have been implemented. Referral to expert centers is therefore a necessity.

GISTs should always be removed, since all of these tumors can potentially metastasize. The laparoscopic and open surgical resection procedure with a "gentle-touch technique" is recommended in order to reduce the risk of hemorrhage and intra-peritoneal dissemination, as GISTs tend to have a friable consistency. Medical treatment with a tyrosine kinase inhibitor (i.e. imatinib) is indicated for recurrent or irresectable GISTs as this treatment has proven very effective, safe and tolerable. Follow-up with CT in patients with GISTs is recommended.

In the presented case, the GIST was excised *in toto* and adrenectomy was performed in the same intervention. Hemostasis was achieved. The following night, acute operation was performed due to hemorrhage, which arose from the adrenectomy area. The recommended follow-up interval for patients with low-risk GISTs like the present case is CT scans every 6 mo.

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Correlation analysis of liver tumor-associated genes with liver regeneration

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Abstract

AIM: To study at transcriptional level the similarities and differences of the physiological and biochemical activities between liver tumor (LT) and regenerating liver cells.

METHODS: LT-associated genes and their expression changes in LT were obtained from databases and scientific articles, and their expression profiles in rat liver regeneration (LR) were detected using Rat Genome 230 2.0 array. Subsequently their expression changes in LT and LR were compared and analyzed.

RESULTS: One hundred and twenty one LT-associated genes were found to be LR-associated. Thirty four genes were up-regulated, and 14 genes were down-regulated in both LT and regenerating liver; 20 genes up-regulated in LT were down-regulated in regenerating liver; 21 up-regulated genes and 16 down-regulated genes in LT were up-regulated at some time points and down-regulated at others during LR.

CONCLUSION: Results suggested that apoptosis activity suppressed in LT was still active in regenerating liver, and there are lots of similarities and differences between the LT and regenerating liver at the aspects of cell growth, proliferation, differentiation, migration and angiogenesis.

Key words: Partial hepatectomy; Rat Genome 230 2.0 Array; Apoptosis; Liver regeneration-associated gene; Liver tumor-associated gene

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INTRODUCTION

The liver is an organ with considerable regenerative capacity[1]. After partial hepatectomy (PH)[2], about 95% of quiescent hepatocytes re-enter synchronously into the cell cycle to replenish the missing hepatocytes[3,4]. Whereas excessive liver mass is regulated by apoptosis[5], this process is called liver regeneration (LR)[3]. The regeneration process, which according to cellular physiological and biochemical activities is divided into the following parts: initiation (0.5-4 h after PH), transition from G0 to G1 (4-6 h after PH), cell proliferation (6-66 h after PH), and cell differentiation and reorganization of the structure-function (72-168 h after PH)[6], or according to time course, into forepart (0.5-4 h after PH), prophase (6-12 h after PH), metaphase (16-66 h after PH) and anaphase (72-168 h after PH), involves various physiological and biochemical activities such as cell activation, de-differentiation, proliferation and its regulation, re-differentiation, and rebuilding of the structure and function[7,8]. Actually, some biological activities in LR including cell proliferation and growth are also observed in liver tumor (LT). It is usually thought that tumorigenesis is mainly ascribed to the anomalous activation of the genes having positive effects on LT cell proliferation, growth, invasion and LT angiogenesis, as well as the genes suppressing LT cell apoptosis, and/or inactivation of the inhibitory genes related to LT cell proliferation, growth, invasion and LT angiogenesis[9], and the promotive genes of LT cell differentiation and apoptosis. To elucidate the intrinsic differences between the two events at transcriptional level, we checked the expression profiles of above genes in regenerating livers following 2/3 hepatectomy utilizing the Rat Genome 230 2.0 Array containing 249 LT-associated genes, and primarily analyzed their expression changes and actions in LR, as well as their relevance with LR.

MATERIALS AND METHODS

Regenerating liver preparation

Healthy Sprague-Dawley rats weighing 200-250 g were

obtained from the Animal Center of Henan Normal University. The 276 rats were separated into 46 groups randomly, 23 hepatectomized groups and 23 sham-operation (SO) groups, and each group included 6 rats. PH was performed according to Higgins and Anderson[2], by which the left and middle lobes of liver were removed. Rats were killed by cervical vertebra dislocation at 0, 0.5, 1, 2, 4, 6, 8, 12, 16, 18, 24, 30, 36, 42, 48, 54, 60, 66, 72, 96, 120, 144 and 168 h after PH and the regenerating livers were observed at corresponding time point. The livers were rinsed three times in PBS at 4℃, and then total 1-2 g livers (100-200 mg livers from middle parts of right lobe of each sample, 6 samples per group) were gathered and mixed together, then stored at -80℃. The SO group was the same as hepatectomized group except the liver lobes were not removed. The laws of animal protection of China were enforced strictly.

RNA isolation and purification

Total RNA was isolated from frozen livers according to the manual of Trizol reagent (Invitrogen Corporation, Carlsbad, California, USA)[10] and then purified base on the guide of RNeasy mini kit (Qiagen, Inc, Valencia, CA, USA)[11]. Total RNA samples were checked to exhibit a 2:1 ratio of 28S rRNA to 18S rRNA intensities by agarose electrophoresis (180 V, 0.5 h). Total RNA concentration and purity were estimated by optical density measurements at 260/280 nm[12].

cDNA, cRNA synthesis and purification

One to eight gram total RNA as template was used for cDNA synthesis. cDNA purification was based on the way established by Affymetrix[13]. cRNA labeled with biotin was synthesized using cDNA as the template, and cDNA and cRNA were purified according to the purification procedure of GeneChip Analysis[13]. Measurement of cDNA, cRNA concentration and purity were the same as above.

cRNA fragmentation and microarray detection

Fifty μL (1 μg/μL) cRNA incubated with 5 × fragmentation buffer at 94℃ for 35 min was digested into 35-200 bp fragments. The hybridization buffer prepared according to the way Affymetrix provided was added to the prehybridized Rat Genome 230 2.0 array produced by Affymetrix, then hybridization was carried out at 45℃ for 16 h on a rotary mixer at 60 rpm. The microarray was washed and stained by GeneChip fluidics station 450 (Affymetrix Inc., Santa Clara, CA , USA). The chips were scanned by GeneChip Scan 3000 (Affymetrix Inc., Santa Clara, CA, USA), and the signal values of gene expression were observed[14].

Microarray data analysis

The normalized signal values, signal detections (P, A, M) and experiment/control (Ri) were obtained by quantifying and normalizing the signal values using GCOS (GeneChip operating software) 1.2[14].

Normalization of the microarray data

To minimize the technical error from the microarray analysis, each sample was hybridized three times to the gene chips. The average value of three measurements was normalized, and statistics and cluster analyses were conducted on these values with GeneMath, GeneSpring (Silicon Genetics, San Carlos, CA) and Microsoft Excel Software (Microsoft, Redmond, WA)[14-16].

Table 1 Primer and probe sequences used to validate the microarray analysis by quantitative RT-PCR

Genes	Primer sequences	Tm	Amplified products
β-actin	FP: CCTGGCACCCAGCACAAT	58℃	221 bp
	RP: GCTGATCCACATCTGCTGGAA	58℃	
	Probe: ATCAAGATCATTGCTCCTCCTGAGCGC	68℃	
jun	FP: TGCAAAGATGGAAACGACCTT	58℃	76 bp
	RP: GCCGTAGGCGCCACTCT	59℃	
	Probe: TACGACGATGCCCTCAACGCCTC	68℃	
myc	FP: CCCCTAGTGCTGCATGAAGAG	59℃	95 bp
	RP: TCCACAGACACCACATCAATTTC	58℃	
	Probe: CACCAGCAGCGACTCTGAAGAAGAACA	68℃	
tp53	FP: ATGAGGCCTTGGAATTAAAGGAT	58℃	98 bp
	RP: CGTAGACTGGCCCTTCTTGGT	59℃	
	Probe: CAGGGCTCACTCCAGCTACCCGAA	68℃	

FP: forward primer; RP: reverse primer.

Verification of array results by RT-PCR

Primer and probe sequences were designed by primer express 2.0 software according to mRNA sequences of three target genes jun, myc, tp53 and internal control ®-actin gene (GenBank number: BC078738, NM_012603, AY009504 and NM_031144) and synthesized by Shanghai GeneCore BioTechnologies Co. Ltd (Table 1).

Identification of genes associated with LR

Nomenclatures such as LT, hepatoma, hepatocellular carcinoma, hepatocarcinogenesis, cholangiocarcinoma and so on were input into the databases at NCBI (www.ncbi.nlm.nih.gov) and RGD (rgd. mcw.edu) to identify rat, mouse and human genes associated with LT. Then these LT-associated genes were reconfirmed through literature searches of the pertinent articles. Besides the rat genes, other genes, that are now thought existing in mouse and/or human and showed a greater than two-fold change in the rat regenerating livers, were referred to as rat homologous genes. Genes that displayed reproducible results with three independent analyses using Rat Genome 230 2.0 array and that showed a greater than two-fold change in expression at least at one time point as a significant difference ($P \leqslant 0.05$) or an extremely significant difference ($P \leqslant 0.01$) between PH and SO, were included as being associated with LR.

RESULTS

Comparison between the quantitative RT-PCR results and the microarray results

The quantitative RT-PCR results of three chosen genes jun, myc and tp53 at 0, 0.5, 2, 4, 6, 12, 24, 30, 36 and 96 h after partial hepatectemy (PH) were compared with Rat Genome 230 2.0 Array results (Figure 1) in order to verify validity of this chip. According to quantitative RT-PCR

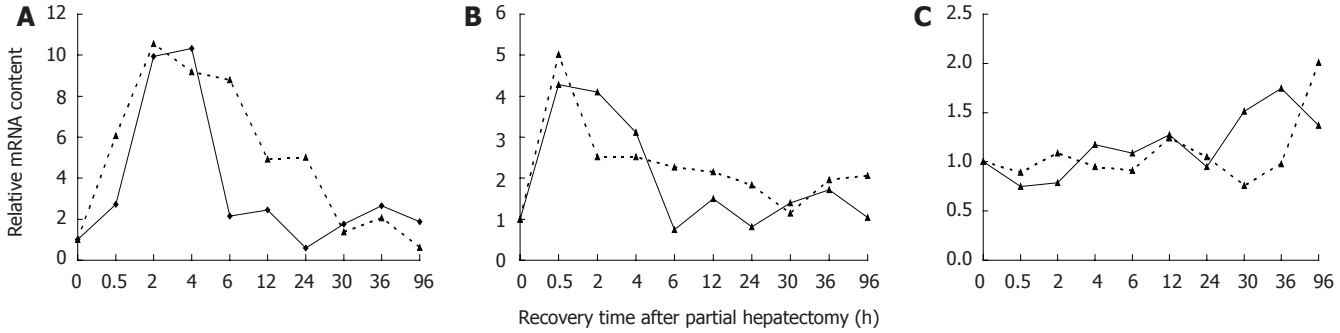

Figure 1 Comparison of relative mRNA levels in regenerating liver detected by Affymetrix Rat Genome 230 2.0 microarray and real-time PCR analysis **A**: myc; **B**: jun; **C**: tp53; Real line represents quantitative real time PCR results; broken line indicates Rat Genome 230 2.0 microarray results.

results, myc was up-regulated at 0.5-12 and 30-96 h after PH with the highest point of 10.33 folds higher than control at 4 h; jun expression was significantly up-regulated at 0.5-4 h after PH, showing the greatest abundance of 4.28-fold of control at 0.5 h; and tp53 was up-regulated at 96 h after PH. The result of RT-PCR suggested that expression profiles of these three genes were basically similar to that of array, which indicated that Rat Genome 230 2.0 Array had great reliability.

Expression changes of the associated-genes in LT and LR

Among 252 genes associated with LT obtained by searching the related data in databases such as NCBI, RGD *etc*., 249 genes were contained in the Rat Genome 230 2.0 Array. 121 of 249 genes yielded meaningful expression changes on at least single time point after PH, showed significant or extremely significant difference between PH and SO, and displayed reproducible results with three independent analyses with Rat Genome 230 2.0 Array, suggesting that these genes were associated with LR. The data listed below indicated that expression trends of 48 genes in LT was similar to that in LR, whereas expression of 34 genes in the former underwent opposite trend comparing with in the latter, and expression changes of 39 genes in LT were similar to that in some time point of LR. Specifically, the same trend towards up regulation of 34 genes and down regulation of 14 genes were exhibited in both LT and LR; 20 up-regulated genes in LT showed down-regulation during LR, and 14 down-regulated genes in LT revealed up-regulation during LR; 23 up-regulated genes and 16 down-regulated genes in LT were up-regulated at some time points and down-regulated at others during LR (Table 2).

The relationship of LT-associated genes with LR

According to function feature and expression profiles of total 121 LT-associated genes in LR, they were divided into six classes and twenty-nine subclasses (Figure 2), and their expression changes in LR were present. Genes up-regulated in both LT and regenerating liver include nine cell proliferation-associated genes (1), four cell growth-associated genes (2), one apoptosis-associated gene (3), nine cell migration-associated genes (4), three angiogenesis-associated genes (5), and eight genes involved in other biological processes (6); Genes down-regulated in both LT and regenerating liver include six cell proliferation-associated genes (7), three apoptosis-associated genes (8), two differentiation-associated genes (9), and three genes with other functions (10); Genes down-regulated in LT but up-regulated in LR include two cell proliferation-associated genes (11), four cell growth-associated genes (12), three apoptosis-associated gene (13), and another five genes having other biological activities (14); Genes up-regulated in LT but down-regulated in LR include six cell proliferation-associated genes (15), two cell growth-associated genes (16), one apoptosis-associated gene (17), three cell migration-associated gene (18), three angiogenesis-associated genes (19), and five genes with other functions (20); Genes up-regulated in LT but up-regulated at some time points and down-regulated at others in LR include four cell proliferation-associated genes (21), three cell growth-associated genes (22), two telomerase-associated genes (23), five cell migration-associated genes (24), and nine genes participating in other actions (25). Genes down-regulated in LT but up-regulated at some time points and down-regulated at others during LR include two cell proliferation-associated genes (26), six cell growth-associated genes (27), one apoptosis-associated gene (28), and seven genes related to biological events differed from the above-mentioned actions (29).

DISCUSSION

Generally, cell proliferation and growth was done in both LT and LR, but the former are malignant, and the latter are controlled stringently. According to our data, proliferation-promoting genes pcna, ccne1, cdk4, ahr, wee1, ccna2, pin1, nek6 and smo[17-22] were up-regulated in both LT and LR, and proliferation-inhibiting genes creb3l3, pten, kit, gjb1, tff1 and csda[23-28], were down-regulated in both, indicating that these genes promote cell proliferation in the two events. Notably, the abundance of CCNA2 mRNA in LT was approximately five-fold higher than that in normal liver[17], and it reached its peak with 45 folds of control at 66 h after PH, which might be associated with an increased proportion of regenerated hepatocytes. Growth-promoting genes hspb1, grn, tgfb1 and serpine1[29-31], whose expression levels were elevated in LT, were up-regulated at metaphase of LR. Among these four genes, serpine1 having the highest expression

Table 2 Expression abundance of 121 liver tumor-associated genes during liver regeneration

Name	Gene Abbr.	Associated to	Fold difference	Comparison LT.	Comparison RRL.
The same in gene expression trend					
Cyclin A2	*Ccna2	2	45.1	↑	↑
WEE1 homolog	Wee1	2	20.9	↑	↑
Cyclin E1	Ccne1	2	18.5	↑	↑
Proliferating cell nuclear antigen	Pcna	2	10.6	↑	↑
Smoothened homolog	Smo	1,2	3	↑	↑
Protein NIMA-interacting 1	Pin1	2	2.5	↑	↑
Cyclin-dependent kinase 4	Cdk4	2	2.5	↑	↑
NIMA (never in mitosis gene a)-related kinase 6	Nek6	2	2.3	↑	↑
Aryl-hydrocarbon receptor	Ahr	1	2.2	↑	↑
Serpin peptidase inhibitor, clade E, member 1	Serpine1	2	16.7	↑	↑
Heat shock 27 kDa protein 1	Hspb1	2	11	↑	↑
Transforming growth factor, beta 1	Tgfb1	2	4	↑	↑
Granulin	Grn	2	2.3	↑	↑
Myeloid cell leukemia sequence 1	Mcl1	2,3	4.3	↑	↑
WNT1 inducible signaling pathway protein 1	Wisp1	2,3	14.9	↑	↑
Selectin E	Sele	2	12.9	↑	↑
Metastasis associated 1	Mta1	2	9.6	↑	↑
TIMP metallopeptidase inhibitor 1	Timp1	2	8.6	↑	↑
Integrin, alpha V	Itgav	2	5.2	↑	↑
Discs, large homolog 7	Dlg7	2	4.3	↑	↑
Lectin, galactoside-binding, soluble, 1	Lgals1	2	3.7	↑	↑
ADAM metallopeptidase domain 17	Adam17	2	2.7	↑	↑
Integrin, beta 1	Itgb1	2	2.6	↑	↑
Calponin 1, basic, smooth muscle	Cnn1	2	7	↑	↑
Macrophage migration inhibitory factor	Mif	2	3.2	↑	↑
Collagen, type XVIII, alpha 1	Col18a1	2	3.1	↑	↑
Connective tissue growth factor	*Ctgf	2	13.9	↑	↑
Hexokinase 2	Hk2	2	8.9	↑	↑
Chemokine (C-C motif) ligand 20	Ccl20	2	8	↑	↑
v-jun sarcoma virus 17 oncogene homolog	*Jun	2	6.9	↑	↑
Methyl-CpG binding domain protein 2	Mbd2	2	3	↑	↑
TERF1 (TRF1)-interacting nuclear factor 2	Tinf2	1	2.8	↑	↑
FMS-like tyrosine kinase 1	*Flt1	2	2.3	↑	↑
Proteasome 26S subunit, non-ATPase, 10	Psmd10	2	2	↑	↑
Phosphatase and tensin homolog	Pten	1,2	0.5	↓	↓
Cold shock domain protein A	Csda	1	0.5	↓	↓
cAMP responsive element binding protein 3-like 3	Creb3l3	1	0.4	↓	↓
v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	Kit	2	0.4	↓	↓
Gap junction protein, beta 1, 32 kDa	Gjb1	1,2	0.2	↓	↓
Trefoil factor 1	Tff1	3	0.1	↓	↓
Caspase 9, apoptosis-related cysteine peptidase	Casp9	2	0.5	↓	↓
Deleted in liver cancer 1	Dlc1	1,2	0.5	↓	↓
B-cell CLL/lymphoma 2	Bcl2	2	0.3	↓	↓
Inhibitor of DNA binding 1	Id1	1,2	0.3	↓	↓
Protein tyrosine phosphatase, receptor type, H	Ptprh	2	0.2	↓	↓
CD74 molecule, major histocompatibility complex, class II invariant chain	Cd74	2	0.4	↓	↓
Hepatocyte growth factor	*Hgf	1,2	0.4	↓	↓
Mannose-binding lectin (protein C) 2, soluble	Mbl2	2	0.2	↓	↓
The contrary in gene expression trend					
Myelocytomatosis oncogene	Myc	1,2	19.7	↓	↑
Sprouty homolog 2	Spry2	2	8.1	↓	↑
Growth arrest and DNA-damage-inducible, beta	Gadd45b	2	55.7	↓	↑
Serine peptidase inhibitor, Kunitz type, 2	Spint2	2	7.2	↓	↑
MAD homolog 4	Smad4	1,2	3	↓	↑
Fibrinogen-like 1	Fgl1	2	2.2	↓	↑
Caspase 8, apoptosis-related cysteine peptidase	Casp8	2	10.6	↓	↑
Interferon gamma	Ifng	1,2	6.5	↓	↑
Tumor protein p53	Tp53	1,2,3	2.9	↓	↑
Early growth response 1	*Egr1	2	18.6	↓	↑
Transcription factor 1, hepatic	Tcf1	2	6.8	↓	↑
O-6-methylguanine-DNA methyltransferase	Mgmt	2	4.3	↓	↑
Glutathione S-transferase theta 1	Gstt1	2	3.2	↓	↑

Glutathione S-transferase M1	Gstm1	2	2.2	↓	↑
Wingless-type MMTV integration site family, member 1	Wnt1	2	0.5	↑	↓
SHC (Src homology 2 domain containing) Transforming protein 1	Shc1	2	0.5	↑	↓
Inhibitor of kappaB kinase beta	Ikbkb	1,2	0.3	↑	↓
FK506 binding protein 4, 59 kDa	Fkbp4	2	0.3	↑	↓
Transcription factor 7-like 2	Tcf7l2	2	0.2	↑	↓
v-erb-b2 erythroblastic leukemia viral oncogene Homolog 2	Erbb2	3	0.1	↑	↓
Heat shock 70kDa protein 1A	Hspa1a	2	0.2	↑	↓
Heat shock 70kDa protein 5	Hspa5	1,2	0.1	↑	↓
High mobility group AT-hook 1	Hmga1	2	0.4	↑	↓
Ras homolog gene family, member C	Rhoc	2	0.3	↑	↓
Cortactin	Cttn	2	0.1	↑	↓
Serpin peptidase inhibitor, clade B, member 3	Serpinb3	2	0.1	↑	↓
Ephrin-B1	Efnb1	2	0.4	↑	↓
Coagulation factor II	F2	1,2	0.3	↑	↓
Trefoil factor 3	Tff3	2	0.3	↑	↓
Forkhead box A2	Foxa2	2	0.4	↑	↓
Glycogen synthase kinase 3 beta	Gsk3b	1,2	0.4	↑	↓
ATP-binding cassette, sub-family B, member 1A	*Abcb1a	1	0.2	↑	↓
Solute carrier family 2 , member 1	*Slc2a1	2	0.2	↑	↓
Apolipoprotein E	*Apoe	2	0.1	↑	↓
The comparable in gene expression trend					
Lysosomal-associated protein transmembrane 4B	Laptm4b	2	2.3,0.5	↑	↑↓
Met proto-oncogene	*Met	1,2,3	2.3,0.4	↑	↑↓
Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105)	Nfkb1	2	2.3,0.4	↑	↑↓
Acyl-CoA synthetase long-chain family member 4	Acsl4	2	2.1,0.4	↑	↑↓
X-box binding protein 1	Xbp1	1	4.3,0.3	↑	↑↓
Nerve growth factor, beta polypeptide	Ngfb	2	3.7,0.5	↑	↑↓
Stearoyl-Coenzyme A desaturase 1	Scd1	1,2	3.5,0.3	↑	↑↓
Telomerase reverse transcriptase	Tert	1,2,3	5.3,0.3	↑	↑↓
Telomeric repeat binding factor (NIMA-interacting) 1	Terf1	1	2.2,0.4	↑	↑↓
Cadherin 17	Cdh17	2	26.1,0.2	↑	↑↓
Glycoprotein (transmembrane) nmb	Gpnmb	2	9.2,0.3	↑	↑↓
Claudin 10	Cldn10	2	6.5,0.3	↑	↑↓
Plasminogen activator, urokinase	Plau	2	3,0.4	↑	↑↓
Secreted phosphoprotein 1	Spp1	2,3	2.7,0.5	↑	↑↓
Alpha-2-macroglobulin	*A2m	2	46.2,0.4	↑	↑↓
Chemokine (C-C motif) receptor 1	Ccr1	2	27.9,0.4	↑	↑↓
Matrix metallopeptidase 9	Mmp9	1,2	9.5,0.5	↑	↑↓
Angiopoietin 1	*Angpt1	2	9.2,0.2	↑	↑↓
Mucin 1, cell surface associated	Muc1	2,3	6.8,0.2	↑	↑↓
Heparanase	*Hpse	2	6.3,0.3	↑	↑↓
Megalencephalic leukoencephalopathy with subcortical cysts 1	Mlc1	2	4.3,0.4	↑	↑↓
Kinase insert domain protein receptor	*Kdr	2	2.4,0.4	↑	↑↓
Prostaglandin-endoperoxide synthase 2	Ptgs2	1,2,3	2.1,0.1	↑	↑↓
Dual specificity phosphatase 1	Dusp1	2	6,0.4	↓	↑↓
Cyclin-dependent kinase inhibitor 1C	Cdkn1c	2	2.8,0.1	↓	↑↓
Growth arrest and DNA-damage-inducible, gamma	Gadd45g	2	8,0.4	↓	↑↓
Hepatic nuclear factor 4, alpha	Hnf4a	2	4.5,0.1	↓	↑↓
Runt-related transcription factor 3	Runx3	1,2	4.3,0.5	↓	↑↓
Insulin-like growth factor binding protein 3	Igfbp3	1,2	2.7,0.4	↓	↑↓
Suppressor of cytokine signaling 3	*Socs3	2	2.5,0.1	↓	↑↓
Suppressor of cytokine signaling 1	*Socs1	1,2	2.4,0.5	↓	↑↓
Fibroblast growth factor 2	Fgf2	1,2	2.1,0.5	↓	↑↓
Fragile histidine triad gene	Fhit	1,2	7.8,0.1	↓	↑↓
Gamma-glutamyltransferase 1	Ggt1	2	3.4,0.2	↓	↑↓
Bone morphogenetic protein 7	Bmp7	2	3,0.4	↓	↑↓
E74-like factor 1 (ets domain transcription factor)	Elf1	2	3,0.4	↓	↑↓
CD80 molecule	Cd80	2	3,0.3	↓	↑↓
Glycine N-methyltransferase	Gnmt	2	2.5,0.4	↓	↑↓
Acyl-Coenzyme A oxidase 1, palmitoyl	Acox1	2	2.3,0.5	↓	↑↓

Asterisks represent the reported genes associated with liver regeneration; LT: liver tumor; RRL: rat regenerating liver; 1: hepatocarcinogenesis; 2: hepatocellular carcinoma; 3: cholangiocarcinoma. ↑ represents genes up-regulated, ↓ down-regulated, and ↑↓ up-regulated at some time points and down-regulated at others during liver regeneration. Gene expression changes in liver tumors were obtained from scientific articles, and expression changes during liver regeneration were the result of microarray detection.

(16.7 folds higher than control) at 6 h following PH might explain why it played an important role in growth of the regenerated hepatocytes. Dysregulated expression of anti-apoptosis gene bcl-2 was present in LT as well as at metaphase and anaphase of LR, and another anti-apoptotic gene mcl1[32], whose change trend toward up-

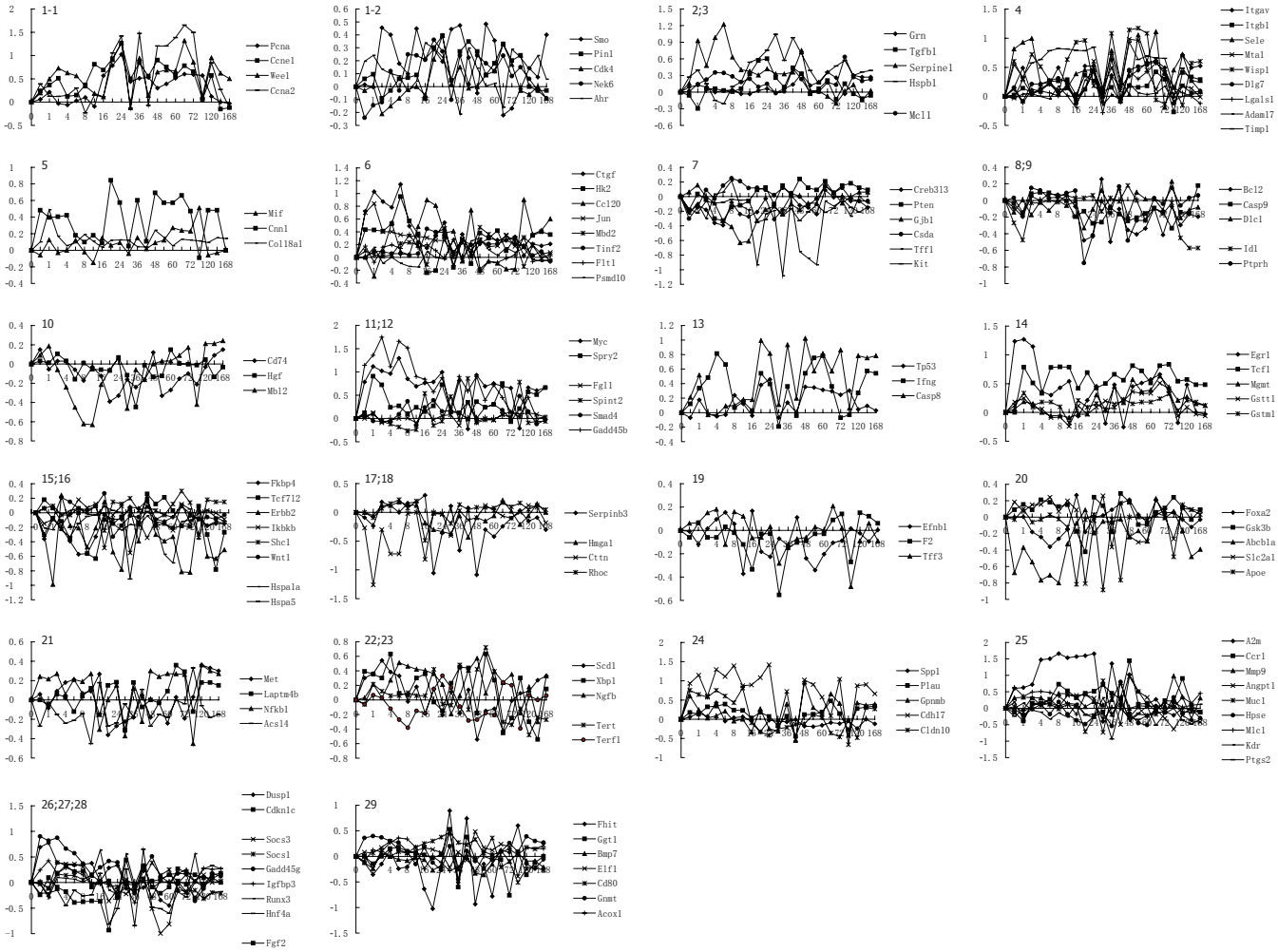

Figure 2 Correlation analysis of 121 liver tumor-associated genes with liver regeneration. Twenty-nine subcategories were obtained by the analysis for detection data of Rat Genome 230 2.0 array with Microsoft Excel. 1-6: 34 genes up-regulated in both liver tumor (LT) and rat regenerating liver (RRL); 7-10: 14 genes down-regulated in both LT and RRL; 11-14: 14 genes down-regulated in LT but up-regulated in RRL; 15-20: 20 genes up-regulated in LT but down-regulated in RRL; 21-25: 23 up-regulated genes in LT were up-regulated at some time points and down-regulated at others in RRL; 26-29: 16 down-regulated genes in LT were up-regulated at some time points and down-regulated at others in RRL. X-axis represents recovery time after PH (h); Y-axis shows logarithm ratio of the signal values of genes at each time point to control.

regulation in LT was identical to that in LR; and down-regulation of pro-apoptosis genes casp9 and dlc1[33,34] occurred in LT and the metaphase of LR, which supported the idea that mcl1, casp9 and dlc1 might are related with cell survival in the two events. The differentiation-related genes id1 and ptprh[35,36] down-regulation in LT and at forepart, metaphase and anaphase of LR suggested that they failed to promote cell differentiation in both events. Up-regulation of enhancement of hepatoma cell migration-related genes itgav, itgb1, adam17, dlg7, sele, mta1, wisp1 and lgals1[37-43] in LT and the entire LR, especially a sustained high-level expression (12-fold higher than control) of wisp1 at 48-60 h post-PH might imply the active cell migration in both LT and LR. According to up-regulated expression pattern in LT and almost the whole LR, metallopeptidase inhibitor timp1 was supposed to perform other biological functions except cell migration in the two events. Mif inducing angiogenesis of LT[44], cnn1 enhancing differentiation of vascular smooth muscle cells[45] and apoptosis-inhibiting gene col18a1 encoding endostatin[46] were up-regulated both in LT and in forepart of LR, which was presumably that the three genes might co-regulate angiogenesis in LT and LR.

Study demonstrated that six pro-proliferation genes ikbkb, shc1, erbb2, fkbp4, wnt1, tcf7l2[47-51] up-regulated in LT were down-regulated during LR, at the same time, the down-regulated genes myc and spry2 possessing anti-proliferation effect[52,53] in LT were up-regulated almost during the whole LR; two growth-promoting genes hspa5 and hspa1a[29,54] and four growth inhibitory genes gadd45b, fgl1, spint2 and smad4[55-58] were respectively up-regulated and down-regulated in LT, whose expression correspondingly underwent opposite trend at metaphase of LR comparing with LT, which was supposed to be closely associated with the differences in proliferation and growth between hepatoma cells and regenerating hepatocytes. Particularly, the expression abundance of gadd45b in human hepatocellular carcinoma was fifteen-fold lower than control[55], just the contrary, its expression reached climax (nearly 56-fold over the control) at 2 h in rat LR, demonstrating there was a significant distinction in gadd45b expression change between normal and transforming liver cells. The down-regulated pro-apoptotic genes tp53, ifng and casp8[59-61] and the up-

regulated apoptosis-inhibitory gene serpinb3[62] in LT were respectively up-regulated and down-regulated at metaphase and anaphase of LR might account for the suppression of apoptosis in LT and enhancement of apoptosis at metaphase and anaphase of LR. In addition, casp8, inactivated caused by frame-shift mutation in hepatocellular carcinoma[61], was up-regulated to its highest levels (10.6 folds higher than control) at 48 h post-PH, and expression of serpinb3 declined to the lowest point (11.4 folds lower than control) at 48 h, signifying the important regulatory effect of the two genes on liver mass. Contribution of efnb1 and tff3 in neovasculargenesis activity[63,64] and the crucial role for f2 in maintenance of vascular integrity[65] were helpful for understanding the hypothesis that the three up-regulated genes in LT down-regulated at metaphase and anaphase of LR implied the control of blood-vessel growth serving as one of modulation pathways of regenerated liver mass. Three hepatoma cell migration and invasion-associated genes hmga1, cttn and rhoc up-regulated in LT[66,67] and down-regulated in LR possibly showed the stronger migration ability of hepatoma cells.

Four up-regulated in LT genes promoting hepatoma cell proliferation including met, laptm4b, nfkb1 and acsl4[68,69], revealed down-regulation at metaphase and up-regulation at anaphase of LR, and another two inhibitory genes dusp1 and cdkn1c[70] were up/down-regulated in LR, i.e. the former was up-regulated at 0.5-12 and 24 h, and down-regulated at 54-60 h, while the latter was down-regulated at 6-18 h and up-regulated at 30 and 42 h; Three up-regulated scd1, xbp1 and ngfb genes involved in hepatoma cell growth in LT[54,71,72] were up-regulated at forepart, prophase and metaphase, and down-regulated at some time points in the late phase of LR, while another six negative regulatory genes including socs1, socs3, gadd45g, igfbp3, runx3 and hnf4a[73-77] down-regulated in LT had a significant increase in expression at some time points and significant decrease at others during LR. The more complicated expression of these genes during the proliferation and growth of regenerating liver cells comparing with that of hepatoma cells concluded from the above results was presumably consistent with the further improved control mechanism upon proliferation and growth of regenerating liver cells than that of hepatoma cells. Telomerase activity of TERT was interfered by terf1 expression product[78], and the two were up-regulated in both LT and the metaphase of LR, while down-regulated at anaphase, indicating that the balance of quantity of the two gene products was essential for maintaining telomere stability. The up-regulated genes spp1, plau and gpnmb promoting hepatoma cell migration and invasion[79,80] as well as the up-regulated intercellular junction-involved cdh17 and cldn10 genes[47,81] were up-regulated at forepart, prophase and some time points after 16h, and down-regulated at other points after 16 h, possibly illustrating that the similar cell migration and interactions in LT occurred at forepart and prophase of LR, however the difference emerged when entering the metaphase of LR. The dysregulated gene fgf2[82] promoting hepatoma cell apoptosis in LT was up-regulated at 4 h post-PH, consistent with the enhanced apoptotic action of regenerating liver cell at the forepart of LR, demonstrating that it acted as a key gene involved in the regulation of apoptosis.

In conclusion, at transcriptional level, while decrease of apoptosis occurring in liver tumors, the process was still going on in LR; as far as cell growth, proliferation, differentiation, migration and angiogenesis are concerned, not only resemblances but differences exist between LT and LR. Especially, expressions of the genes, such as ccna2, serpine1, wisp1, gadd45b, casp8 and serpinb3, display marked changes in two events, so their actions deserve the further study. Of course, the process of DNA →mRNA→protein→function could be influenced by many factors including gene mutation, protein interaction etc. Therefore, the further analyses are required for confirming the above results using techniques such as gene addition, knock-out, RNAi, *etc.*

COMMENTS

Background

The liver is susceptible to tumorigenesis, and it's also an organ with strong regenerating capacity. Both hepatocarcinogenesis and LR are associated with cell proliferation and growth.

Research frontiers

Most research works had been done on the mechanism of hepatocarcinogenesis and LR, but heretofore no research report was found in investigating the correlation between them.

Innovations and breakthroughs

121 genes associated with both hepatocarcinogenesis and LR were found. Their expression changes in LR were analyzed by Rat Genome 230 2.0 Array, and their expression similarities and differences in hepatocarcinogenesis and LR were compared.

Applications

Our results may provide basic and useful data for further research on both LT therapy and LR mechanism.

Terminology

PH model, an effective surgical operation to trigger LR, was established by Higgins and Anderson in 1931. The hepatectomized rat, whose left and middle lobes of liver were removed, can recover its liver mass after about a week, so it's widely used to investigate LR mechanism.

Peer review

This is an important work examining common genes associated with HCC and LR.

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Establishing models of portal vein occlusion and evaluating value of multi-slice CT in hepatic VX2 tumor in rabbits

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Abstract

AIM: To establish models of portal vein occlusion of hepatic VX2 tumor in rabbits and to evaluate the value of multi-slice CT.

METHODS: Forty New Zealand rabbits were divided into 4 groups according to digital table: Immediate group (group A; transplantation of tumor immediately after the portal vein occlusion), 3-wk group (group B; transplantation of tumor at 3 wk after the portal vein occlusion), negative control group (group C) and positive control group (group D), 10 rabbits in each group. Hepatic VX2 tumor was transplanted with abdominal-embedding innoculation immediately after the portal vein occlusion and at 3 wk after the portal vein occlusion. Meanwhile, they were divided into negative control group (Left external branch of portal vein was occluded by sham-operation, and left exite was embedded and inoculated pseudoly) and positive control group (Transplanted tumor did not suffer from the portal vein occlusion). All rabbits were scanned with multi-slice CT.

RESULTS: All 40 animals were employed in the final analysis without death. Tumor did not grow in both immediate group and 3-wk group. In 3-wk group, left endite was atrophied and growth of tumor was inhibited. The maximal diameter of tumor was significantly smaller than that in positive control group (2.55 ± 0.46 *vs* 3.59 ± 0.37 cm, t = 5.57, P < 0.001). Incidences of metastasis in the liver and lung were lower in 3-wk group than those in positive control group (10% *vs* 40%, and 90% *vs* 100%, respectively). The expression intensities of the vascular endothelium growth factor (VEGF) in groups A, B, C and D were 0.10 ± 0.06, 0.66 ± 0.21, 0.28 ± 0.09 and 1.48 ± 0.32, respectively. VEGF expression level in the test group A was significantly lower than that in the negative control group C (t = 5.07; P < 0.001). In addition, VEGF expression in the test group B was significantly lower than that in the positive control group D (t = 6.38; P < 0.001). Scanning with multi-slice CT showed that displaying rate of hepatic artery branches was obviously lower in grade Ⅲ (40%) than that in grade Ⅰ (70%) and Ⅱ (100%) (P < 0.05); but there was no significant difference in displaying rate of the portal vein at various grades. Values of blood flow (BF) of the liver, blood volume (BV), mean transit time (MTT) and permeability of vascular surface (PS) were lower in the immediate group and 3-wk group than those in control groups, but values of hepatic arterial fraction (HAF) were increased. Significant positive correlations were existed between BF and BV (r = 0.905, P < 0.01), and between BF and PS (r = 0.967, P < 0.01), between BV and PS (r = 0.889, P < 0.01). A significant negative correlation existed between PV and HAF (r = -0.768, P < 0.01), between PS and HAF (r = -0.557, P < 0.01). The values of BF, BV and PS had a positive correlation with VEGF (r_{BF} = 0.842, r_{BV} = 0.579, r_{PS} = 0.811, P < 0.01) . However, there was no significant correlation between the values of MTT and HAF and the VEGF expression (r_{MTT} = 0.066, r_{HAF} = -0.027).

CONCLUSION: Ligating the left external branch of portal vein is an ideal way to establish models of portal vein occlusion in rabbits with hepatic VX2 tumor. Multi-slice CT plays a key role in evaluating effect of portal vein occlusion.

Key words: Portal vein; Multi-slice CT; X-ray computer; VX2 tumor; Portal vein occlusion model

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INTRODUCTION

Clinical researches have found that portal vein occlusion is beneficial to inhibit growth of hepatocarcinoma, promote compensatory hyperplasia of un-blocking hepatic tissue and decrease metastasis of portal vein occlusion. However, it should be further proved by animal experiments[1,2]. This

study was designed to investigate the models of portal vein occlusion of hepatic VX2 tumor in rabbits and to evaluate value of multi-slice CT.

MATERIALS AND METHODS

Materials

The experiment was carried out in Department of Radiology of Xinqiao Hospital of the Third Military Medical University of Chinese PLA from July 2004 to July 2006. Forty New Zealand rabbits of both genders, weighing 2.0-3.0 kg, were provided by Experimental Animal Center of the Third Military Medical University of Chinese PLA [Certification: SYXK (army) 2004-031]. All animals were divided according to digital table into 4 groups: Immediate group (group A; transplantation of tumor immediately after the portal vein occlusion), 3-wk group (group B; transplantation of tumor at 3 wk after the portal vein occlusion), negative control group (group C) and positive control group (group D), each group consisting of 10 animals.

Methods

Establishment of experimental models: VX2 cancer was inoculated into the muscles in the inguinal region. Three or 4 wk later, the rabbits with VX2 epidermoid carcinoma developed in the legs were anesthetized with 30 mg/kg soluble pentobarbitone. Then, under sterile condition, tumor was stripped to obtain hoary fish-meat-like tissue near to envelope. The sample tissues were put into sterile saline to remove necrosis tissue and fiber tissue and cut into pieces of 1.0-2.0 mm^3 with eye scissors.

Experimental methods: Rabbits were divided into four groups as follows: (1) Immediate group (group A): After anesthesia, all limbs of rabbits were fixed on domestic operative table at supine position. Under all aseptic precautions, a 2-cm midline incision in the epigastric region was given, and the liver was exposed by opening the abdomen in layers. Left exite of the liver was exposed and origin of the left external branch of the portal vein was bluntly separated; and then, the proximal and distal tips were ligated and blocked. Color of the left liver was observed; when color of the left external liver was darkened, the left external branch of the portal vein was blocked completely (Figure 1A and B). Hepatic VX2 tumor was transplanted with abdominal-embedding innoculation. Eye forceps were used to cut hepatic tissue near the thick part of left exite. A tumor mass was transplanted into a flask-like incision with 3-5-mm mouth and 5-8-mm fundus, gelatin sponge was used for hemostasis, and aperture was purse-string sutured. Then the exposed liver was returned to the abdominal cavity and abdomen was closed in layers. Multi-slice CT was done 3 wk later. (2) 3-wk group (group B): Hepatic VX2 tumor was transplanted with abdominal-embedding innoculation as aforementioned. Three weeks later, the left external branch of portal vein of successfully transplanted samples was blocked completely with the same method mentioned above. Multi-slice CT was done 2 wk later. (3) Negative control group (group C): Left external branch of portal vein was done sham-operative block, and left exite was embedded and inoculated pseudoly. (4) Positive control group (group D): Left external branch of portal vein underwent sham-operation block and other processes were the same as in the 3-wk group.

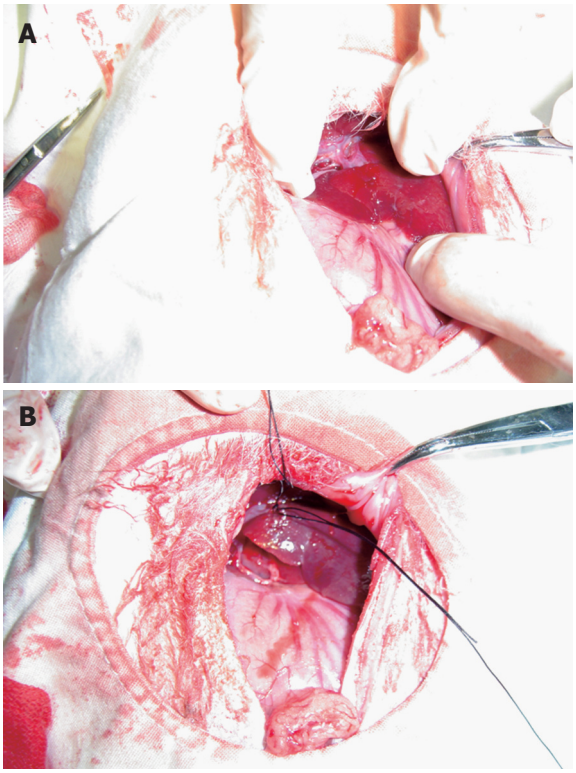

Figure 1 **A**: The left exite and left endite branch of portal vein were separated naturally in the immediate group; **B**: Eye forceps was used to cut hepatic tissue near the thick part of left exite after the ligature of left exite branch of portal vein.

Multi-slice CT examination: Rabbits were fasted for 12 h and detained needle (24 GA × 0.75 IN) was inserted into ear-border vein before scanning. GE lightspeed 16 CT scanning apparatus and MEDRAO Vistron CTTM hypertensive injection syringe were used for examination. After anesthesia, rabbits were fixed on board at supine position and pricked their abdominal bandage to reduce movement artifact. Plain scan of multi-slice CT was performed using the parameters as follows: 80 kV tube voltage, 80 kV tube current, 512 × 512 matrix, and 2.5-mm section thickness. For vascular imaging of multi-slice CT, 4-6 mL of Ioversol, a contrast medium [Optiray, Mallinckrodt Medicine Co. Ltd., USA, 320 g(I)/L], was administered with hypertensive injection syringe at 2.0 mL/s; meanwhile, samples were scanned from head to foot and from diaphragm to 2 cm below the liver. Scanning parameters included thickness of selective by 2.5 mm, thickness of rebuilding by 1.25 mm, rebuilding interval 0.65 mm, successively scanning with 16-row detector, bed shift 27.5 mm with one convolution of stock, ratio of snail space 1.325:1, non-tilt scan, tube voltage 80 kV, tube current 130 kV, matrix 512 × 512 and scanning sight 12-16 cm. Arterial phase scanning started 7 s after injection of contrast medium; 13 s during portal venous phase; 50 s during delayed phase. Plain scan of multi-slice CT perfusion: Four close layers of tumor were regarded as the perfused layers; 1 mg/kg anectine chloride was

pushed with detained needle; and Toggling-table technique of multi-slice CT was used to scan the four layers during respiratory depression; 4 mL of contrast medium was injected with injection syringe at 2.0 mL/s and scanning started 1 s after the injection. Figures were collected as film pattern for 50 s. Scanning parameters were as follows: Thickness of selective by 2.5 mm/4i, thickness of rebuilding by 5 mm, successively scanning with 16-row detector, bed shift 27.5 mm with one convolution of stock, ratio of snail space 1.325:1, non-tilt scan, tube voltage 80 kV, tube current 130 kV, matrix 512 × 512 and scanning sight 12-16 cm.

Post-processing after multi-slice CT: Three scanning figures were transmitted to AW4.2 workstation by local network. Figures during periods of the hepatic artery and portal vein scanning were selected from each rabbit for recovery of volume, projection of maximal density and multi-planar reformation. On the basis of target vessels, volume rendering was convoluted and cut at 360° through regulating width of window, position of window, brightness and diaphaneity. Projection of maximal density was obtained on the basis of volume rendering. According to course of vessels, all sections were rebuilt planarly but coronal site was mainly reconstructed. Statistics of branch of the hepatic artery showed that the proper hepatic artery was regarded as branch Ⅰ, left and right hepatic arteries as branch Ⅱ, and other hepatic arteries as ≥ branch Ⅲ. Similarly, trunk of portal vein was regarded as branch Ⅰ, left and right portal veins as branch Ⅱ, and other portal veins as ≥ branch Ⅲ. Volume rendering, projection of maximal density and multi-planar reformation were combined with each other during multi-slice CT scanning, and samples with the best quality and the most branches were included. Data were dealt with perfusion 3.0 software package with deconvolution method, and blood flow (BF) of the liver, blood volume (BV), mean transit time (MTT), permeability of capillary vessel surface (PS) and fraction of hepatic arterial fraction (HAF) were calculated to obtain perfusing figures of each parameter.

Seldinger's technique was adopted to puncture the femoral artery and superior mesenteric vein of the alive rabbits after MSCT perfusion scan, and 3F micro-catheter was used to catheterize rabbit's hepatic artery to perform hepatic arteriography. Direct portography was performed *via* the superior mesenteric vein at the time and after hepatic arteriography. In all rabbits, hepatic artery and portal vein perfusions were performed by injecting a contrast medium suspension containing barium sulfate and saline (1:3). The displaying rates of the Ⅰ, Ⅱ and ≥ Ⅲ branches of the hepatic artery and the portal vein with multi-slice CT angiography (MSCTA), digital subtraction angiography (DSA) and vascular perfusion techniques were calculated, respectively, especially the ligature of the branch of portal vein.

The body weight, metastasis and maximum diameter of the implanted tumor were observed and measured at autopsy of the all rabbits. To match with the lesion seen on the MSCT perfusion imaging, the partial tumors were taken out for pathological examination. All specimens were stained with hematoxylin and eosin (HE), and VEGF expression in the tumors was examined using immunohistochemistry. The correlation between the parameters of MSCT perfusion and the staining intensity of VEGF was analyzed using a computer-assisted image analyzer.

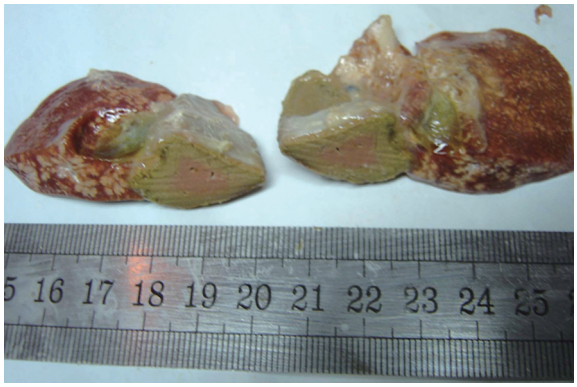

Figure 2 Tumor did not grow in the immediate group after 3 wk. Volume of left exite was decreased, nature quality was hard and color was deep.

RESULTS

Pathological results of the experimental animals

All 40 animals were utilized in the final analysis without any loss. Animals in the immediate group and negative control group had normal behaviors before death, including appearance, activity, diet, body mass and hair. Tumor did not grow in the immediate group after 3 wk. The left exite showed a decreased size with hard consistency (Figure 2). In addition, left exite was adherent to left endite or stomach wall; however, volumes of other hepatic lobes were bigger in group A than that in negative control group. In 3-wk group, left endite was atrophied and growth of tumor was inhibited; and the maximal diameter of tumor was smaller than that in positive control group.

Tumor grew successfully with a rate of 100% (20/20) both in 3-wk group and positive control group after inoculation of hepatic VX2 tumor. Except 4 animals in 3-wk group and 10 in positive control group, who were found to have indifferent mood, bradykinesia, reductive diet, decreased body mass and disheveled hair before death, other 6 in 3-wk group showed normal behaviors. In 3-wk group, tumors were hoar, and 8 animals had necrosis of tumor tissue (Figure 3). In positive control group, tumors were hoar, hard with expansive growth, and 4 rabbits had necrosis of tumor tissue. Numerous tumor-induced vessels were observed and envelope was not obvious. Examination of corpses showed that volume of left endite was decreased, hepatic tissue was shrunk and growth of tumor was inhibited obviously in 3-wk group, and maximal diameter of tumor was markedly smaller than that in positive control group ($t = 5.57$, $P < 0.001$). Incidences of metastasis in the liver and lung were insignificantly lower in 3-wk group than those in positive control group (Table 1). Metastasis in the mesentery and omentum appeared as swollen lymph node; metastasis in the diaphragm muscles appeared as widespread nod-like guava seed, especially in

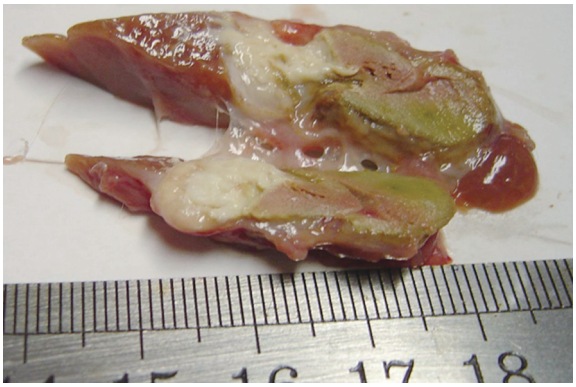

Figure 3 Necrosis of tumor tissue with liquefaction was shown in group B after 2 wk.

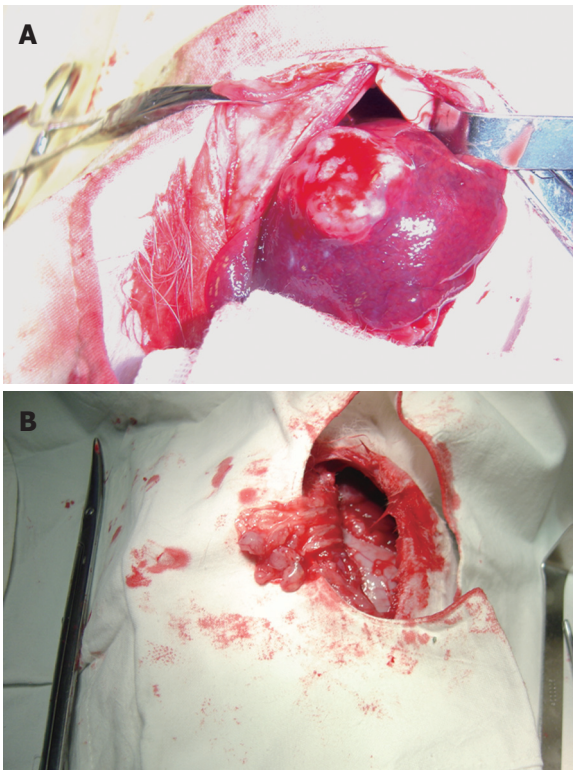

Figure 4 **A**: Tumor of positive control group was hoar, hard and expansively grown; **B**: The metastasis of mesentery was shown as swollen lymph node in the same rabbit of Figure 4A.

Table 1 Results of examination of corpses in 3-wk group and positive control group (n = 10)

Group	Maximal diameter (cm)	Metastasis in liver (n/%)	Metastasis in lung (n/%)	Close metastasis (n)
3-wk	2.55 ± 0.46[b]	1/10[b]	4/10[a]	7
Positive control	3.59 ± 0.37	10/10	9/10	9

[a]$P < 0.05$, [b]$P < 0.001$, *vs* positive control group.

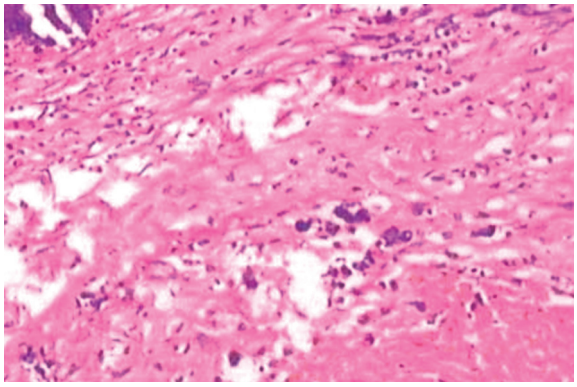

Figure 5 The liver tissue of the left external lobe underwent coagulation necrosis, and was stained homogenously red with hematoxylin-eosin stain in group A (HE, × 100).

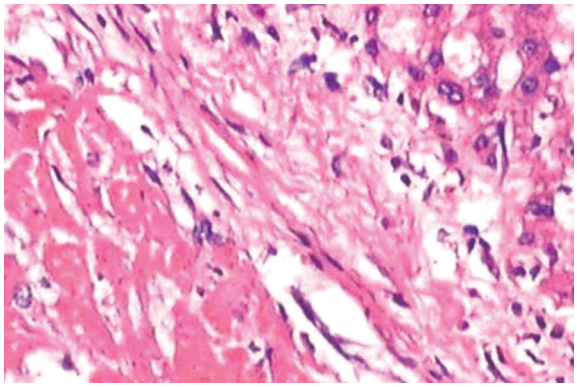

Figure 6 The histologic appearance of the necrosis of tumor tissue included marked proliferation of collagen fibers in group B (HE, × 100).

the left diaphragm; metastasis in the bilateral lungs appeared as widespread hoar granulations (Figure 4A and B). Uprightness hydroperitonia of chest was observed in 5 animals of 3-wk group and 9 of positive control group.

On microscopic examination 3 wk after the portal branch ligation, no tumor was found to grow in any rabbits of group A. The left external lobe shrank, became harder, appeared darker in color, and sealed by adhesions to the left internal lobe or to the stomach. In contrast, the other liver lobes of rabbits in group A were increased in size as compared with those in group C. The liver tissue of the left external lobe underwent coagulation necrosis, and was stained homogenously red using hematoxylin-eosin staining (Figure 5). The histologic appearance of these lesions of group B included marked proliferation of collagen fibers, among which only scattered tumor cells were noted in 6 cases. The tumors were completely necrotic, and no viable tumor cell was seen under microscope in 2 out of 10 rabbits of group B (Figure 6). The remaining 2 rabbits had more survival tumors with many small necrosis foci. The expression intensities of the VEGF in groups A, B, C and D were 0.10 ± 0.06, 0.66 ± 0.21, 0.28 ± 0.09 and 1.48 ± 0.32, respectively (Figures 7 and 8). VEGF expression level in the test group A was significantly lower than that in the negative control group C (t = 5.07; $P < 0.001$). Moreover, VEGF expression level in the test group B was significantly lower than that in the positive control group D (t = 6.38; P <0.001) (Table 2).

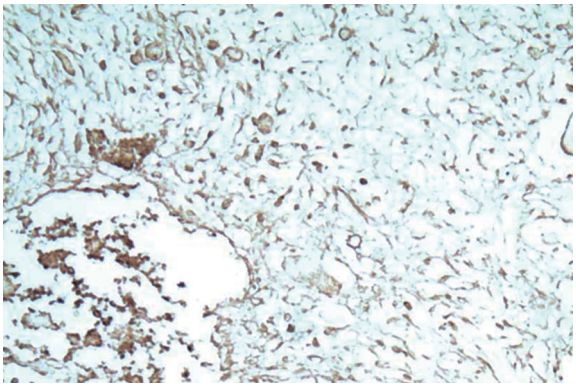

Figure 7 The average optical density (AOD) of VEGF in group A was 0.09 ± 0.08 (SP, × 100).

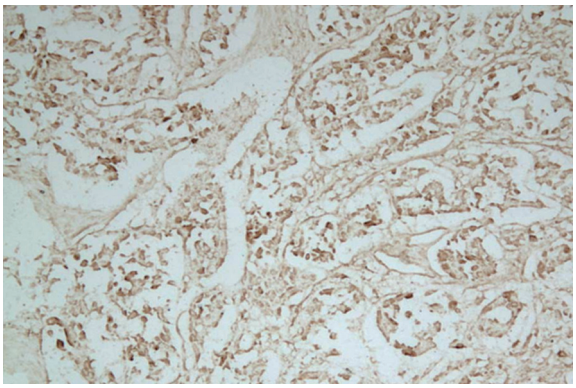

Figure 8 The AOD of the VEGF in group B was 0.72 ± 0.28 (SP, × 100).

Table 2 The expression intensity of VEGF in groups A, B, C and D (n = 10)

Group	VEGF	t	P
Group A	0.10 ± 0.06	5.07	0.00
Group C	0.28 ± 0.09		
Group B	0.66 ± 0.21	6.38	0.00
Group D	1.48 ± 0.32		

Table 3 The success rate of MSCTA, DSA and vascular perfusion of the hepatic artery and portal vein (n/%)

	MSCTA	DSA	Vascular perfusion
Hepatic artery	100% (40/40)	85% (17/20)[1]	97.3% (36/37)
Portal vein	100% (40/40)	95% (19/20)	97.4% (38/39)

[1] The success rate of MSCTA and DSA of hepatic artery, $P < 0.05$.

Results of the hepatic vascular branch with MSCTA, DSA and vascular perfusion

The success rates of MSCTA, DSA and vascular perfusion of the hepatic artery and portal vein are shown in Table 3 and Figure 9. The success rate of hepatic arterial examination by DSA was significantly lower than that by MSCTA (85% *vs* 100%, $P < 0.05$), but there was no significant difference in the other examinations of the

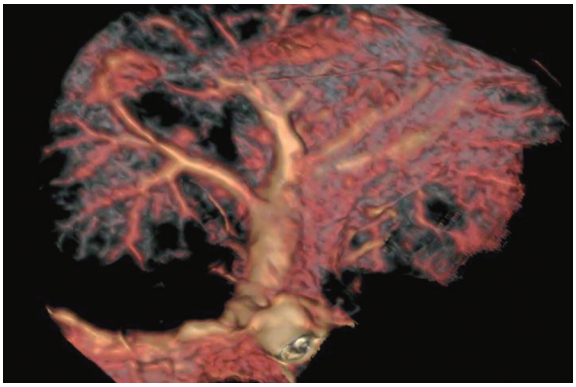

Figure 9 The volume rendering image of MSCTA shows of the normal portal vein.

Table 4 Comparisons of vascular-imaging displaying rate of MSCTA, DSA and vascular perfusion of hepatic artery [%(n/n)]

Grade	MSCTA (n = 40)	DSA (n = 17)	Vascular perfusion (n = 33)
Grade Ⅰ	100 (40/40)	100 (17/17)	97.0 (32/33)
Grade Ⅱ	70 (28/40)	100 (17/17)	90.9 (30/33)
⩾ Grade Ⅲ	40 (16/40)	88.24 (15/17)	75.77 (25/33)

Table 5 Comparisons of vascular-imaging displaying rate of MSCTA, DSA and vascular perfusion of portal vein [%(n/n)]

Grade	MSCTA (n = 40)	DSA (n = 19)	Vascular perfusion (n = 38)
Grade Ⅰ	100 (40/40)	100 (19/19)	100 (38/38)
Grade Ⅱ	100 (40/40)	94.7 (18/19)	100 (38/38)
⩾ Grade Ⅲ	100 (40/40)	89.5 (17/19)	100 (38/38)

hepatic artery and portal vein. As shown in Table 4, the total displaying rate of the branch ⩾ Ⅲ of the hepatic artery by MSCTA (40%) was significantly lower than that of Ⅰ and Ⅱ branches of the hepatic artery (100% and 70%, respectively) ($P < 0.05$), and was significantly lower than that by DSA and the vascular perfusion (88.24% and 75.77%, respectively) ($P < 0.01$). However, there was no obvious difference in the displaying rate of the portal vein among examinations by MSCTA, DSA and the vascular perfusion (Table 5). Scanning with multi-slice CT showed that the displaying rate of the hepatic artery branches was markedly lower in grade Ⅲ than that in grade Ⅰ and Ⅱ ($P < 0.05$); but there was no significant difference in displaying rates of the portal vein among various grades. The imaging shows distortion and tenuity of the hepatic artery. The results proved that vessels were not formed at collateral portal vein (Figure 10A-D). Branches of the portal vein of normal rabbits were widely distributed and the border was clear.

MSCT perfusion in the experimental animals

The time-density curve (TDC) of the abdominal aorta and portal vein showed rapid rise and drop, fast rise and drop, respectively. The TDCs of liver parenchyma in negative control group C showed slow up and down, while there

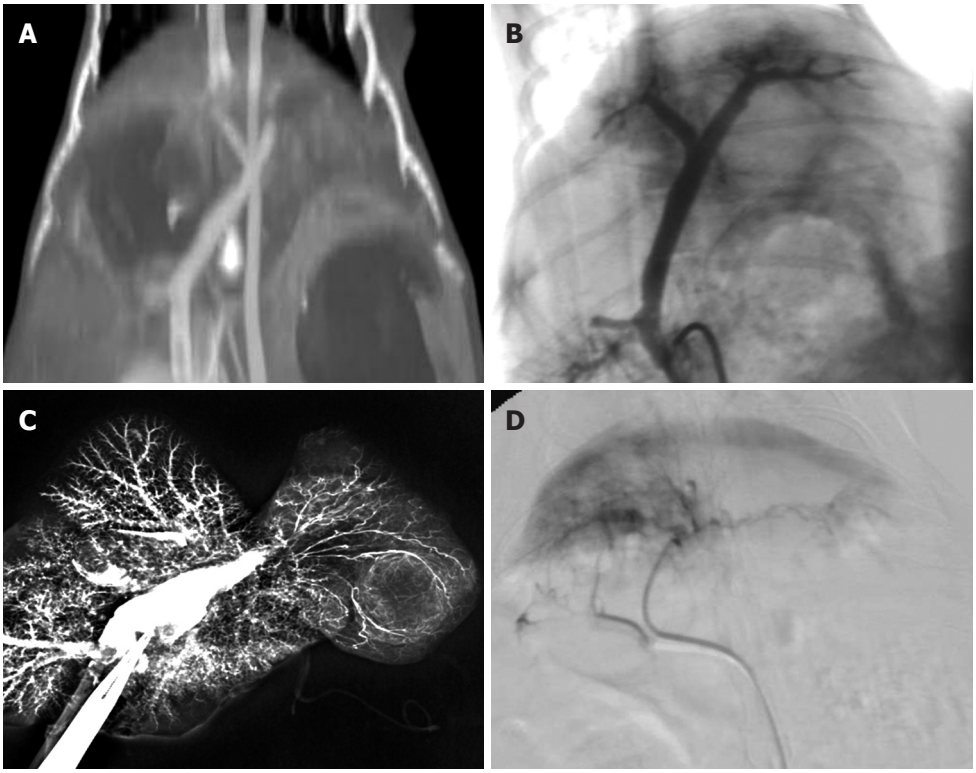

Figure 10 **A**: The multiplanar reformation image shows the ligature of the left external branch of portal vein; **B**: The DSA of the portal vein shows the ligature of the left external branch of portal vein; **C**: The vascular perfusion of both hepatic artery and portal vein shows the blood supply of the left external tumor from left hepatic artery; **D**: DSA of hepatic artery shows the blood supply of the left external tumor from left hepatic artery.

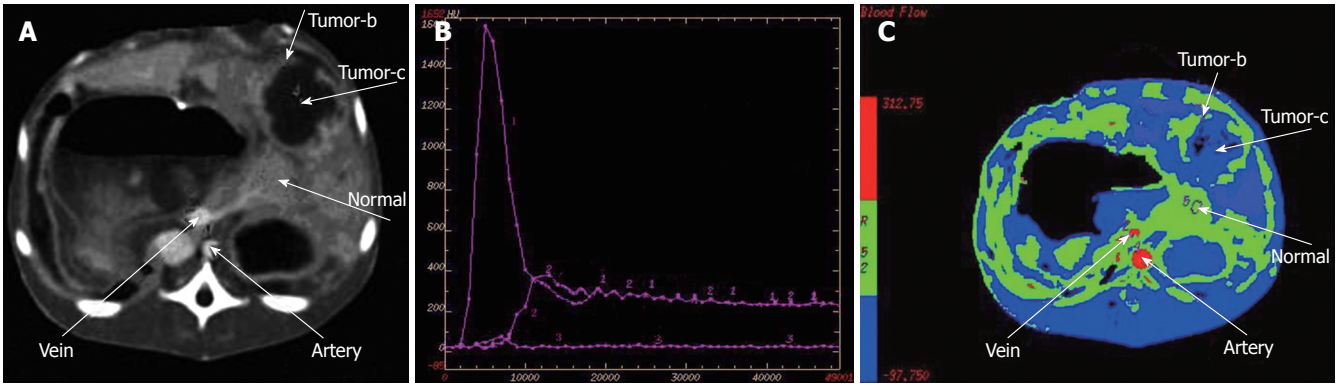

Figure 11 **A**: MSCT scanning shows that hepatic tissue and tumor wall of left exite were shrunk in group of portal vein occlusion at 3 wk after tumor transplantation; **B**: The TDC of hepatic tumor of the left external lobe in the same rabbit of Figure 4A; **C**: The blood flow image in the same rabbit of Figure 11A.

Table 6 Comparison of parameters of multi-slice CT (mean ± SD, n = 10)

CT-perfused parameter	Group A	Group B	Group C	Group D
Blood flow in per 100 g hepatic tissue (mL/min)	1.40 ± 0.70	48.76 ± 7.31	133.21 ± 14.42[b]	296.53 ± 39.62[d]
Blood volume in per 100 g hepatic tissue (mL)	0.33 ± 0.17	3.33 ± 0.53	28.77 ± 3.32[b]	30.64 ± 3.32[d]
Mean transit time (s)	4.33 ± 1.41	14.7 ± 1.66	11.67 ± 0.58[b]	6.79 ± 0.85[d]
Permeability of vascular surface in per 100 g hepatic tissue (mL/min)	0.15 ± 0.18	11.71 ± 2.33	22.10 ± 4.39[b]	36.16 ± 5.91[d]
Hepatic arterial infusion	0.99 ± 0.03	0.99 ± 0.02	0.25 ± 0.06[b]	0.63 ± 0.01[d]

[b]$P < 0.01$ *vs* immediate group; [d]$P < 0.01$ *vs* 3-wk group.

was a slight enhancement of liver parenchyma of the left external lobe in the test group A, which was washed out quickly. In the positive control group D, the hepatic tumor enhanced intensely and washed out quickly, while the TDCs of the hepatic tumor in the test group B showed rapid rise and drop. Comparison of parameters of multi-slice CT is shown in Table 6. In the immediate group and 3-wk group, values of blood flow of the liver, blood volume, mean transit time and permeability of vascular surface were decreased but values of HAF were increased than those in the control groups (Figures 11 and 12).

Significant correlations between various MSCT per-

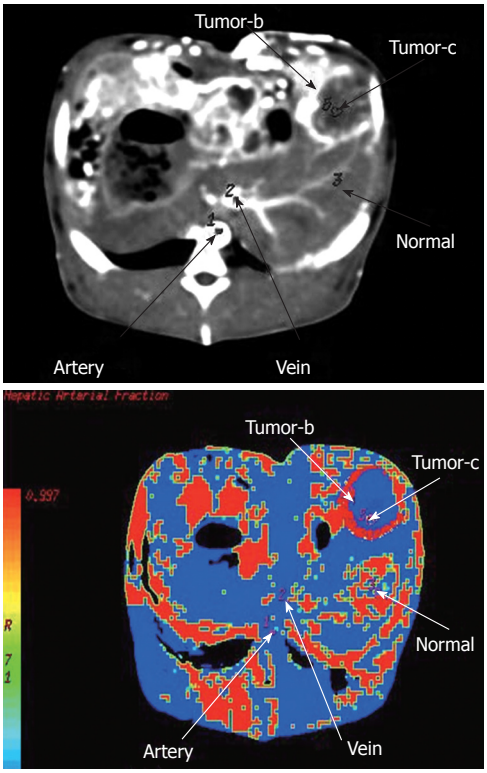

Figure 12 **A**: MSCT scanning shows the hepatic VX2 tumor of the left external lobe in a rabbit of positive control group; **B**: HAF image in the same rabbit of Figure 12A.

fusion parameters were observed in this study (Table 7). A significant positive correlation existed between BF and BV (r = 0.905, P < 0.01), between BF and PS (r = 0.967, P < 0.01), and between BV and PS (r = 0.889, P < 0.01). A significant negative correlation existed between PV and HAF (r = -0.768, P < 0.01), and between PS and HAF (r = -0.557, P < 0.01). The values of BF, BV and PS had a positive correlation with VEGF (r_{BF} = 0.842, r_{BV} = 0.579, r_{PS} = 0.811, P < 0.01). However, no significant correlation of MTT and HAF with VEGF was observed (r_{MTT} = 0.066, r_{HAF} = -0.027).

Table 7 Coefficient correlation of MSCT perfusion parameters and VEGF (r)

Parameters	BV	MTT	PS	HAF	VEGF
BF	0.905[b]	-0.132	0.967[b]	-0.494	0.842[b]
BV		-0.057	0.889[b]	-0.768[b]	0.579[b]
MTT			0.044	-0.157	-0.066
PS				-0.557[b]	0.811[b]
HAF					-0.027

[b]P < 0.01.

DISCUSSION

If trunk of the portal vein of rats or dogs was ligated once, animals would die within several hours due to congestion or hemorrhage. However, Gaub *et al*[3] suggested that 90% portal vein was ligated, 14% animals died, and other rats could survive by compensation of the rest 10% hepatic tissue. During our pre-experiment, posterior branches of the portal vein of two rabbits were maintained, and they died within 24 h. When left branches were ligated, behaviors such as mood, activity and diet of the experimental rabbits were poorer than those of sham-operation rabbits in the first 4 d after operation, and the symptoms recovered within the next 5 d. However, after ligation of left exite, the behaviors were not changed remarkably. In this study, we found that the portal vein consisted of thick superior mesenteric vein, inferior mesenteric vein and thin splenic vein. About 1.0-2.0 cm away from the right posterior branch, a thin caudate lobe was branched. Right anterior branch and left internal branch of the portal vein were circled by hepatic tissue; therefore, it was not an ideal site for ligation. However, left exite and left endite were separated naturally. Partial left exites were located below the xiphoid process and easy to inoculate.

In this study, left external branch of the portal vein of 24 rabbits was not circled by hepatic tissue, and that of remaining 16 rabbits was circled by a little hepatic tissue but it was easy to separate. Twenty rabbits underwent portal vein ligation, but no one died during the operation. So we thought that ligation of left external branch might be an ideal model to study on portal vein occlusion. Some researches showed that complete proximal occlusion by ligation of right branch of the portal vein was similar to distal occlusion by a mixture of lipiodol and ethanol to cause liver lobes atrophy, thereby suggesting that ligation of branch of the portal vein could simulate portal vein occlusion well, completely block the liver lobes dominated by target branches and avoid the experimental effects caused by hematological diffusion of contiguous portal vein. Ligation of left external branch was beneficial to investigate the effect of portal vein occlusion on blood supply of hepatic VX2 tumor. In our study, the portal vein occlusion at the time of tumor implantation could completely inhibit the growth of hepatic VX2 tumor in rabbits. Moreover, the portal vein occlusion after 3 wk of the tumor implantation could effectively inhibit the growth of hepatic VX2 tumor in rabbits.

Vascular-imaging displaying rate of multi-slice CT mainly included volume rendering (VR), maximum intensity projection (MIP) and multiplanar reformation (MPR)[4-7]. VR used nearly complete data to remain primitive relationship of spatial dissection; otherwise, quality of image was good, third dimension was strong, and artifact was few. However, vessels which needed to reconstruct were different from density of peripheral tissue. MIP was imaged with the maximal density of data, and the applied rate was less than 10%. Reconstructed time was short, and it could reflect difference of density, distinguish calcification and reinforcement of vessels; however, third dimension was poor and spatial relationship was not satisfied. MPR was processed with planar technique of spontaneous coronal and arrowed sections; horizontal axial surface could show complex anatomic structure. However, it did not have third dimension; therefore, it should be combined with VR and MIP to observe course of target vessels. The combinations of VR,

MIP and MPR could well show branches and course of the hepatic artery and portal vein, direct reflect details of spatial dissection, and surely evaluate blocking effect of the portal vein.

TDCs can directly reflect the characteristics of blood flow of hepatic VX2 tumor after the portal vein occlusion in rabbits. The BF, BV and PS of the MSCT perfusion parameters are well correlated with the expression intensity of the VEGF[8,9]. MSCT perfusion imaging may reflect the features of blood perfusion to evaluate the hemodynamic change in hepatic VX2 tumor after the portal vein occlusion in rabbits. Re-perfused CT scanning could directly reflect blood supply of local tissue and changes of hemodynamics through blood flow of liver, blood volume, mean transit time, permeability of vascular surface and fraction of HAF[10-22]. Total re-perfused volume was the blood flow in local region within unit time and was derived from initial value of impulse residue function. Blood flow was expressed as IRF which was represented as summarization of the hepatic artery and portal vein. Results in this study suggested that blood flow was the highest in positive control group, which was related to thicker tumor vessels or more accepted contrast medium. However, the decreasing values in other three groups caused blocked blood stream of the portal vein.

Mean transit time is the time of blood stream from artery to vein and calculated by immediate rejection of sham contrast medium and the first moment of IRF. The first moment of curve was expressed as $y = f(t)$ which was determined as average value of t. Each t value was added according to f (t) to consist of total value of $t \times f(t)$, and then, divided total value of f (t), which was represented as IRF. The calculation was complex; therefore, it was not analyzed in details. Mean transit time of portal vein occlusion was shorter in the immediate group than that in negative control group. The reasons were that hepatic tissue of left exite was shrunk, necrotized and fibrosed after ligation and produced a few interstitial microvessels so as to inhibit slow circulation of hepatic sinuses of normal hepatic tissue from artery to vein and accelerate blood stream through interstitial microvessels[23]. Otherwise, mean transit time of the portal vein occlusion was longer in the 3-wk group than that in the positive control group. Circulation in hepatic tumor under normal condition was from the hepatic artery, sham sinusoid, portal vein, hepatic vein (or artery to short circulation of portal vein) to inferior vena cava[24-26]. When branches of the portal vein were ligated, contrast medium was deposited in tumor; therefore, mean transit time would be prolonged, especially showing a significant difference between the hepatic artery and short circulation of portal vein in positive control group.

Blood volume meant distribution of contrast medium in unit hepatic tissue. It was mainly affected by vascular bed of tumor and status of internal blood. In this study, blood volume was the highest in the positive control group due to more vascular beds and slow blood stream in vascular pool or vascular lake which could accept more contrast medium. However, blood volume was lower in the immediate group and 3-wk group than that in the control groups due to the inhibition of contrast medium in the portal vein.

Permeability of vascular surface pointed to one-way transmission rate of contrast medium from blood capillary to intercellular space. The effective factors were permeability of local microvascular wall and pressure difference of lumen of microvessels. In this study, permeability of vascular surface was the highest in the positive control group due to dysplasia of vascular endothelium, uncompleted basement membrane and increased vascular permeability. There was large amount of blood supply in tumoral artery and the pressure in it was higher; however, due to portal vein occlusion, plenty of fiber tissue was produced in hepatic tissue or tumor so as to cause decrease of pressure difference in the immediate group and 3-wk group. In addition, atrophy of hepatic tissue and inhibition of tumor growth could also decrease blood flow in the liver; therefore, dosage of contrast medium was decreased in intercellular space per unit time.

HAF pointed to percentage of blood volume supplied by the hepatic artery and ranged from 0 to 1. In this study, HAF nearly reached 1 in the immediate group and 3-wk group after ligation, and this was related to blood volume supplied by other arteries except the hepatic artery.

In conclusion, ligating left external branch of the portal vein is an ideal way to establish models of portal vein occlusion in rabbits with hepatic VX2 tumor. Furthermore, multi-slice CT plays a key role in evaluating effect of portal vein occlusion.

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BASIC RESEARCH

Reversal of hyperglycemia in diabetic rats by portal vein transplantation of islet-like cells generated from bone marrow mesenchymal stem cells

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Abstract

AIM: To study the capacity of bone marrow mesenchymal stem cells (BM-MSCs) trans-differentiating into islet-like cells and to observe the effect of portal vein transplantation of islet-like cells in the treatment of streptozotocin-induced diabetic rat.

METHODS: BM-MSCs were isolated from SD rats and induced to differentiate into islet-like cells under defined conditions. Differentiation was evaluated with electron microscopy, RT-PCR, immunofluorescence and flow cytometry. Insulin release after glucose challenge was tested with ELISA. Then allogeneic islet-like cells were transplanted into diabetic rats *via* portal vein. Blood glucose levels were monitored and islet hormones were detected in the liver and pancreas of the recipient by immunohistochemistry.

RESULTS: BM-MSCs were spheroid adherent monolayers with high CD90, CD29 and very low CD45 expression. Typical islet-like cells clusters were formed after induction. Electron microscopy revealed that secretory granules were densely packed within the cytoplasm of the differentiated cells. The spheroid cells expressed islet related genes and hormones. The insulin-positive cells accounted for 19.8% and mean fluorescence intensity increased by 2.6 fold after induction. The cells secreted a small amount of insulin that was increased 1.5 fold after glucose challenge. After transplantation, islet-like cells could locate in the liver expressing islet hormones and lower the glucose levels of diabetic rats during d 6 to d 20.

CONCLUSION: Rat BM-MSCs could be transdifferentiated into islet-like cells *in vitro*. Portal vein transplantation of islet-like cells could alleviate the hyperglycemia of diabetic rats.

Key words: Bone marrow mesenchymal stem cells; Trans-differentiation; Islet; Insulin; Transplantation

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INTRODUCTION

Islet transplantation has recently been shown to be an efficient therapy for type 1 diabetic patients. However, immune rejection, recurrent autoimmune attack against transplanted islets and the lack of donor islets restrict its application in clinical practice[1]. Alternatively, much effort has been made to use the renewable source of stem cells[2-5]. The development of a simple, reliable procedure to obtain autologous stem cells capable of differentiating into functional insulin-producing cells for transplantation would alleviate the major limitations of islet availability and allogeneic rejection. Recent studies have shown that embryonic stem cells, hepatic oval cells and pancreatic stem cells could be differentiated into pancreatic islet-like cells *in vitro* and *in vivo*[6-8]. However, these sources are still not enough to provide abundant autologous stem cells. In contrast, bone marrow (BM) has been known to be a safe and abundant source for large quantities of adult stem cells. Some data have revealed that stem cells derived from BM are capable of being reprogrammed to become functional insulin-producing cells and normalize hyperglycemia in streptozotocin-induced diabetic mice and rats by renal subcapsular transplantation[9-11]. The current article reports a potential procedure to generate islet-like cells from BM mesenchymal stem cells (BM-MSCs) by high glucose, nicotinamide and exendin-4. After transplantation

via portal vein, allogeneic islet-like cells could locate in the recipient's liver, expressing islet hormones and alleviate the hyperglycemia of diabetic rats.

MATERIALS AND METHODS

Isolation and cultivation of BM-MSCs

Sprague-Dawley (SD) rats of closed colony were purchased from Animal Center, Nanjing Medical University. All the procedure was accordant with animal experiment guidelines of the university. BM was obtained from the femurs and tibias of 10 male SD rats (200-250 g) under aseptic condition, separated by Ficoll density gradients centrifugation and dispersed into a single cell suspension. BM cells (1×10^6 cells/mL) were cultured in 75 cm^2 flask with low glucose (5.6 mmol/L) Dulbecco's modified eagle's medium (LG-DMEM, Gibco, USA) containing 10% fetal bovine serum (FBS, Hyclone, USA), HEPES (20 mmol/L), L-glutamine (2 mmol/L), penicillin (100 μ/mL) and streptomycin (100 mg/mL) at 37℃ in a humidified atmosphere of 95% air and 5% carbon dioxide. Suspended cells were disposed 24 h later and adherent cells were cultured in 10% FBS LG-DMEM which was changed every 3 d. BM-MSCs gaining 80%-90% confluence were passaged by digestion with 0.25% trypsin and 0.02% EDTA. Following two to three passages, the cells became morphologically homogeneous.

Flow cytometric analysis

After the third passage, BM-MSCs were released by trypsinization. The cells were incubated with anti-rat phycoerythrin (PE)-labeled CD45 antibody (1:20) and fluorescein isothiocyanate (FITC)-labeled CD90 antibody (1:20) (Caltag, USA) or FITC-labeled CD29 antibody (1:20) (Biolegend, USA) for 20 min, then resuspended in 1% paraformaldehyde/PBS and acquired onto FACSCalibur (BD, USA), the positive rates were assessed by Cellqust software. Isotypematched rat immunoglobulins served as controls for autofluorescence.

In vitro differentiation cultures

At the third passage, BM-MSCs with 80% confluence were induced to differentiate into pancreatic islet cells. Cells were induced with 5% FBS HG-DMEM (25 mmol/L glucose) for 14 d, and added 10 mmol/L nicotinamide (Sigma, USA) for 7 d, and then 10 nmol/L exendin-4 (Sigma) for 7 d.

Converted Microscopy and Electron Microscopy

During differentiation, morphological changes of BM-MSCs were investigated under a converted microscope. BM-MSCs and differentiated cells (D-MSCs) were fixed in 5% glutaraldehyde for 2 h at 4℃, washed in PBS, transferred to 1% osmic acid for 2 h at 4℃, washed in PBS, then dehydrated in acetonic acid and embedded. Ultra thin sections were counterstained using uranyl acetate and lead citrate, then viewed by electron microscope (JEM-1010, Japan).

Detection of Islet related gene expressions by RT-PCR

Total RNA from pre-induced BM-MSCs, D-MSCs and normal rat pancreas tissue was isolated using TRIzol reagent (Gibco) and pretreated with DNase to remove genomic DNA contamination. Transcriptional gene expressions related to pancreatic endocrine development and function were determined by RT-PCR kit (Promega, USA). GAPDH was used as an internal control. PCR cycles were as follows: initial denaturation at 95℃ for 5 min, followed by 30 cycles of 95℃ for 30 s, annealing temperature (Tab 1) for 30 s, 72℃ for 30 s, and final extension at 72℃ for 10 min. PCR products were separated by electrophoresis in 1.0% agarose gels and photographed by Kodak digital camera. The name and sequences of the primers, the sizes of PCR products, and annealing temperature for each pair are listed in Table 1. The primers were synthesized by Shanghai BIOASIA Biologic Technology CO. LTD.

Observation of Islet hormones expressions by Immunofluorescence

Pre-induced BM-MSCs, D-MSCs and RIN-m5F cells were grown in plastic six-well plates on slide coverslips (22 × 22 mm^2). Cells were fixed in methanol for 15 min, washed with PBS, incubated with 0.01% Triton-100 and first antibody for 20 min, washed with PBS, and cultured with secondary antibody for 20 min, washed with PBS. Insulin, C-peptide, glucagons (Gcg), somatostatin (SS) and islet amyloid polypeptide (IAPP) expressions were observed under laser confocal microscope (LSM510, Carl Zeiss, Germany). First antibody: guinea pig anti-insulin (1:50) (Zymed, USA), rabbit anti-Gcg (1:50) (Zymed), rabbit anti-SS (1:50) (Zymed), rabbit anti-IAPP (1:50) (Zymed), goat anti-rat C-peptide (1:50) (Linco Research Inc., USA); Secondary antibody: anti-Guinea pig IgG (1:20) FITC conjugated (KPL,USA), anti-rabbit IgG (1:20) FITC/Cy5 conjugated (KPL), anti-goat IgG (1:20) FITC conjugated (KPL).

Analysis of Insulin expression by Flow cytometry

Pre-induced BM-MSCs and D-MSCs (n = 10) were fixed in methanol for 15 min, washed with PBS, incubated with 0.01% Triton-100 and Guinea pig anti-Insulin (1:50) for 20 min, washed with PBS, and cultured with anti-Guinea pig IgG FITC conjugated (1:20) for 20 min, washed with PBS, then resuspended in 1% paraformaldehyde/PBS solution and acquired onto FACSCalibur. The insulin expression and mean immunofluorescence intensity were assessed by Cellqust software. Isotypematched rat immunoglobulins served as controls for autofluorescence.

Measurement of Insulin secretion by ELISA

Pre-induced BM-MSCs and D-MSCs (10^6/mL, n = 5) were switched to serum-free LG-DMEM containing 0.5% BSA for 12 h, washed twice with PBS, then stimulated by HG-DMEM for 2 h. The culture medium was collected and frozen at -70℃. Serum-free LG-DMEM containing 0.5% BSA was used as a control for secreted insulin measurement. Insulin release was detected by rat insulin enzyme-linked immunosorbent assay (ELISA kit, Linco) according to the manufacturer's instructions.

Table 1 List of rat gene-specific primers in RT-PCR

Genes	Forward primer	Reverse primer	Annealing temperature (℃)	GenBank accession no.	Size of PCR product (bp)
InsulinI	CCGTCGTGAAGTGGAG	CAGTTGGTAGAGGGAGCAG	57	NM_019129	156
InsulinII	ATGGCCCTGTGGATCCGCTT	CTAGTTGCAGTAGTTCTCCA	53	NM_019130	333
Glucagon	ATCATTCCCAGCTTCCCAGA	CGGTTCCTCTTGGTGTTCAT	54	NM_012707	152
Somatostatin	CAGGAACTGGCCAAGTAC	AGTTCTTGCAGCCAGCTTTG	54	NM_012659	187
IAPP	AGTCCTCCCACCAACCAATGT	AGCACAGGCACGTTGTTGTA	54	NM_012586	220
GLUT-2	TTACTCTCCATTTCAGTCCTTTGT	TAGAGCAGCTCTTTATTCCAGATTT	53	J03145	165
GK	ACCAGAAAGGGGAGGCCT	ATTAAAAACTCCCCCACAGTCC	51	NM_012565	179
GLP-1R	TCCTGTTAAAGCTGCAAGGC	TTGTCCGAGAGGAAGGCTG	54	NM_012728	232
PDX-1	GGTGCCAGAGTTCAGTGCTAA	CCAGTCTCGGTTCCATTCG	53	NM_022852	249
Ngn3	CTTCACAAGAAGTCTGAGAACACCAG	CTGCGCATAGCGGACCACAGCTTC	57	NM_021700	233
NeuroD1	TGTCGTTACTGCCTTTGGAA	CGATCTGAATACAGCTACACGAA	53	NM_019218	151
PAX-6	CGACAAGATTTGCCATGGAT	CAACCTTTGGAAAAACCAACA	54	NM_013001	179
Nkx2.2	CACGCAGGTCAAGATCTG	TGCCCGCCTGGAAGGTGGCG	55	X81408	188
GAPDH	CACCCTGTTGCTGTAGCCATATTC	GACATCAAGAAGGTGGTGAAGCAG	57	NM_017008	196

IAPP: Islet amyloid polypeptide; GK: Glucokinase.

Transplantation of islet-like cells to STZ-induced diabetic rats

Hyperglycemia was induced in 16 male SD rats of closed colony (Body weight 180-200 g) through intraperitoneal injection of 60 mg/kg of streptozotocin (STZ). Blood glucose levels were determined using Roche ACCU-CHEK glucose meter. Stable hyperglycemia (blood glucose levels ranging between 16.7-33.3 mmol/L) developed in 14 rats one week later. Under general anesthesia, the rats received a transplant of 5×10^6 D-MSCs ($n = 7$) or pre-induced BM-MSCs ($n = 3$) or 600 freshly isolated islets ($n = 4$) *via* portal vein. Glucose levels were monitored by tapped tail-vein blood under non-fasting condition every two days after transplantation for 28 d.

Observation of D-MSCs grafts by Immunohistochemical examination

Liver and pancreas of the recipient at d 12 after D-MSCs transplantation were removed and fixed in 4% formaldehyde and embedded in paraffin. To detect D-MSCs grafts in the liver, all liver lobes were sampled. Sections were stained with anti-insulin (1:50) (DAKO, Denmark), anti-Gcg (1:50) (Zymed), anti-SS (1:50) (Zymed) and anti-IAPP (1:50) (Zymed), respectively. Immunohistochemical analysis was performed using an EnVision™ + System-HRP (DAB) (DAKO) following the manufacture's instruction.

Statistics analysis

Data are presented as mean ± SD. Results were analyzed using one-way ANOVA. Statistical significance was set at $P < 0.05$.

RESULTS

Morphological changes during BM-MSCs differentiation

Pre-induced BM-MSCs were typical of spindle and fibrocyte-like adherent monolayers (Figure 1A) with high CD90 positive rate (96.3% ± 1.3%), CD29 positive rate (93.9% ± 0.8%) and very low CD45 expression (0.3% ± 0.4%) (Figure 1F). When being switched into 5% FBS HG-DMEM, BM-MSCs began to form three dimensional, islet-like clusters (Figure 1B). After induced by nicotinamide and exendin-4, clusters were increased and some half suspended in the culture medium (d = 80-200 μm) (Figure 1C). An electron micrograph of D-MSCs revealed structures typical of a secretory cell, including rough endoplasmic reticulum, Golgi complex, a few large vacuoles and secretory vesicles containing dense granules (Figure 1E). However, few could be found within the cytoplasm of pre-induced BM-MSCs (Figure 1D).

Gene expressions of BM-MSCs and D-MSCs

Transcriptional gene expressions related to pancreatic endocrine development and function were not detected in pre-induced BM-MSCs. However, when BM-MSCs were induced with 5% FBS HG-DMEM for 14 d, insulin (Ⅰ and Ⅱ), Gcg, SS, IAPP, glucagon-like peptide (GLP)-1 receptor (GLP-1R), pancreatic duodenal homeobox-1 (PDX-1), Ngn3, NeuroD1, PAX-6 and GLUT-2 messages were positively expressed. After adding nicotinamide and exendin-4 for another 14 d, D-MSCs showed the expression of the aforementioned genes and GK, Nkx2.2 mRNAs (Figure 2).

Proteins analysis of BM-MSCs and D-MSCs

To investigate the expressions of pancreatic islet hormones, immunofluorescence analyses were performed for insulin (INS), C-peptide, Gcg, SS and IAPP in D-MSCs. RIN-m5F cells were shown to strongly express INS, C-peptide, Gcg, SS, but negative for IAPP (Figure 3A). Pre-induced BM-MSCs were negative for the above islet hormones (Figure 3B); However, D-MSCs did express these cytoplasmic proteins (Figure 3C); Some D-MSCs co-expressed INS/Gcg, INS/SS, INS/IAPP (Figure 3D). Flow cytometry showed that the insulin positive rate of

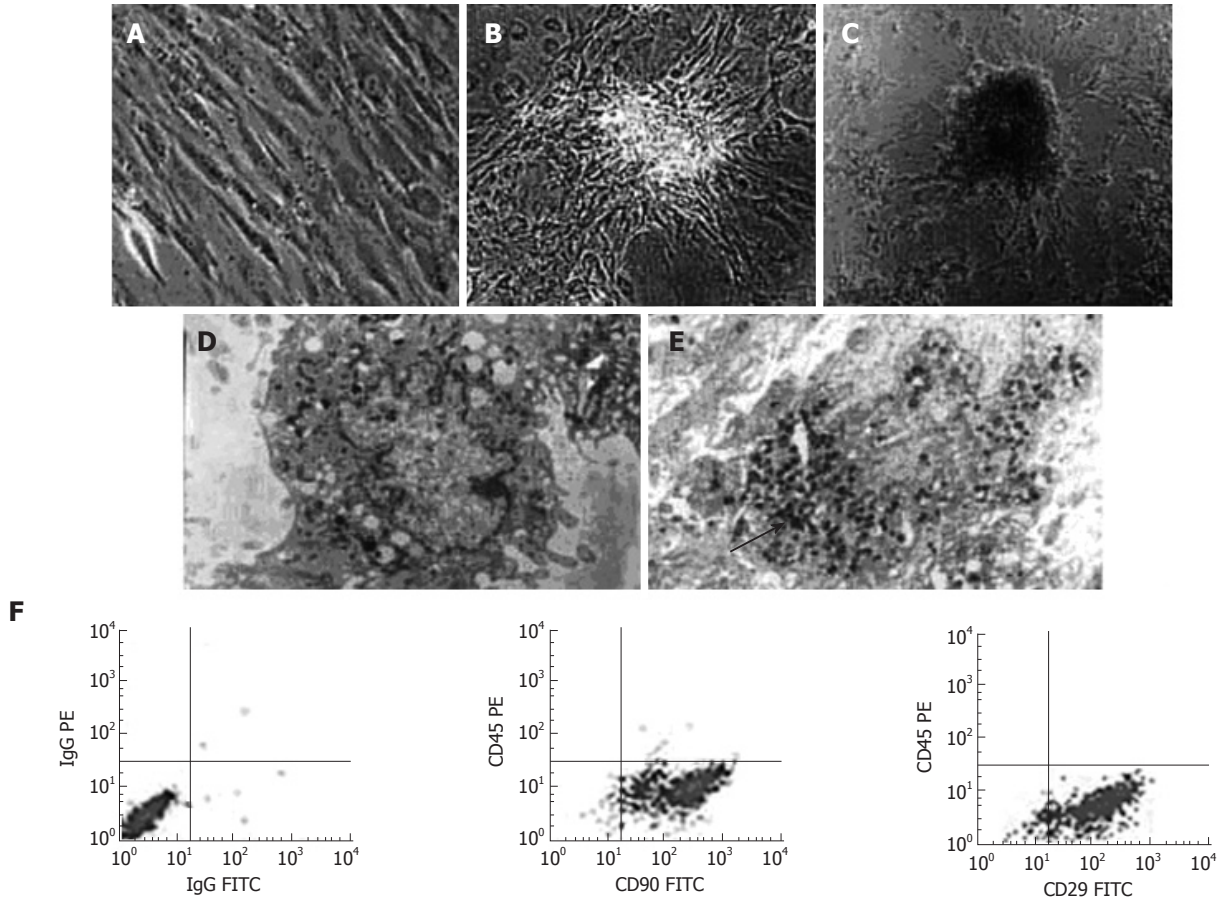

Figure 1 Morphological changes of BM-MSCs during differentiation. **A**: The third passage of pre-induced BM-MSCs (× 500); **B**: BM-MSCs formed islet-like clusters under 5% FBS HG-DMEM culture (× 500); **C**: Some clusters were half suspended in the culture medium after induced by nicotinamide and exendin-4 (× 500); Electron microscopy: Secretory granules (the black arrow shows) are densely packed within the cytoplasm of D-MSCs (**E**, × 4000) whereas few is found in pre-induced BM-MSCs (**D** × 5000); **F**: Surface markers of BM-MSCs showed that CD90 and CD29 positive rates were more than 93% whereas CD45 expressions were less than 1%.

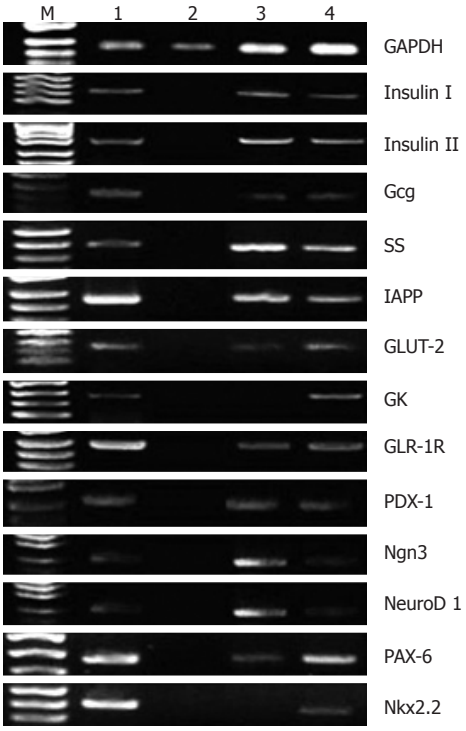

Figure 2 Gene expressions during BM-MSCs differentiation. M DNA marker: 1 Rat pancreas; 2 Pre-induced BM-MSCs; 3 BM-MSCs in 5% FBS HG-DMEM culture; 4 BM-MSCs in 5% FBS HG-DMEM culture with nicotinamide and exendin-4.

cells induced by 5% FBS HG-DMEM for 14 d was about 11.2%. When added with nicotinamide and exendin-4, the insulin positive rate was up to around 19.8% and mean fluorescence intensity was increased by 2.6 folds, which was significantly higher than that of pre-induced BM-MSCs (Figure 4, $P < 0.05$). To determine whether D-MSCs could secret insulin and response to a glucose challenge, insulin release from pre-induced BM-MSCs and D-MSCs was measured by ELISA. Pre-induced BM-MSCs and cells induced with 5% FBS HG-DMEM for 14 d showed little insulin secretion and glucose response. However, D-MSCs could secret a small amount of insulin (roughly 2.0 ng/10^6 cells) into medium and increase by 1.5 fold in the presence of glucose challenge (Figure 4).

Reversal of hyperglycemia in STZ-induced diabetic rats

To determine whether D-MSCs possessed the capacity to correct hyperglycemia in diabetic rats, D-MSCs (5×10^6/rat) were transplanted *via* portal vein into STZ-induced diabetic rats. Pre-induced BM-MSCs (5×10^6/rat) and freshly isolated islets (600/rat) were transplanted as controls. As demonstrated in Figure 5, glucose levels in D-MSCs implanted rats began to decrease at d 6 after transplantation, kept below 15 mmol/L during d 12 to d 16, and then elevated again after d 20. In contrast, glucose levels in islets implanted rats decreased at d 2,

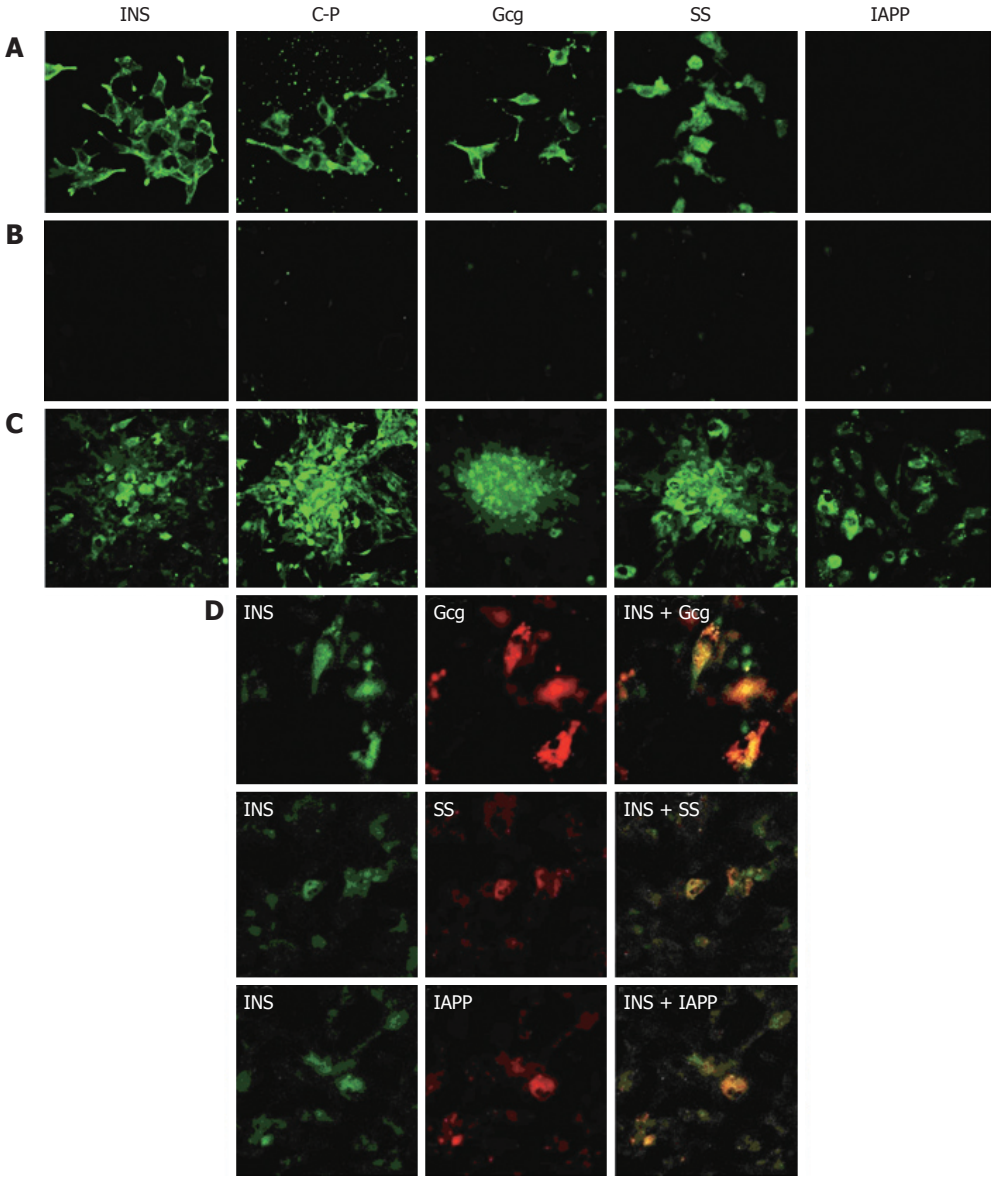

Figure 3 Immunofluorescence reveals BM-MSCs differentiated into islet-like cells *in vitro*. Rat RIN-m5F cells displayed insulin (INS), C-peptide (C-P), glucagons (Gcg), somatostatin (SS), but without islet amyloid polypeptide (IAPP) (**A**, × 400); Pre-induced BM-MSCs were negative for the above islet hormones staining (**B**, × 400); D-MSCs showed the staining of INS, C-P, Gcg, SS, IAPP (**C**, × 400); Some D-MSCs co-expressed INS/Gcg, INS/SS, INS/IAPP (**D**, × 400).

kept below 15 mmol/L during d 4 to d 8, and then elevated again after d 10. Glucose levels in the pre-induced BM-MSCs implanted rats remained elevated ($P < 0.05$). Immunohistochemical analysis revealed that D-MSCs grafts were located in the recipient's liver in close proximity to the portal vein and expressed insulin, Gcg, SS and IAPP (Figure 6B). Very few native islets could be found in the pancreas of STZ-induced diabetic recipient rats. In the remaining pancreatic islets, only few insulin-expressing cells located in the center, rich Gcg-expressing cells setting at the periphery, a few SS-expressing cells and IAPP-expressing cells scattering in the islets (Figure 6A).

DISCUSSION

In the present study, we generated pancreatic islet-like cells from BM-MSCs under an *in vitro* differentiation procedure promoted by nicotinamide and exendin-4, and confirmed the presence of insulin production by RT-PCR, immunofluorescence, electron microscopy, glucose stimulating insulin secretion test. After transplantation *via* portal vein, allogeneic islet-like cells could locate in the recipient's liver expressing islet hormones and alleviate the hyperglycemia of diabetic rats.

The mammalian pancreas arises initially as dorsal and ventral buds that emanate from the embryonic foregut endodermal layer and differentiates into the endocrine cells forming the pancreatic islets of Langerhans under a cascade of gene activation events controlled by transcription factors including PDX-1, Ngn3, NeuroD1, PAX-6, PAX-4, Nkx2.2, Nkx6.1 and so on[12,13]. Inducing stem cells to differentiate into islet-like cells resembles this reprogrammed process. This strategy has been successfully applied in inducing embryonic stem cells, hepatic oval cells and pancreatic stem cells into pancreatic islet-like cells in vitro under defined condition[6-8]. However, these sources are still not suitable for clinical application. BM harbors large quantities of adult stem cells that could be easily obtained. Among them, BM-MSCs are multipotent and can differentiate into lineages of mesenchymal tissues, endodermal and epidermal cells, such as tendon, muscle, adipocytes, chondrocytes, osteocytes, vascular endothelial cells, neurocytes, lung cells and hepatocytes[14-19]. Moreover, BM-MSCs are of great multiplication potency. Cell-

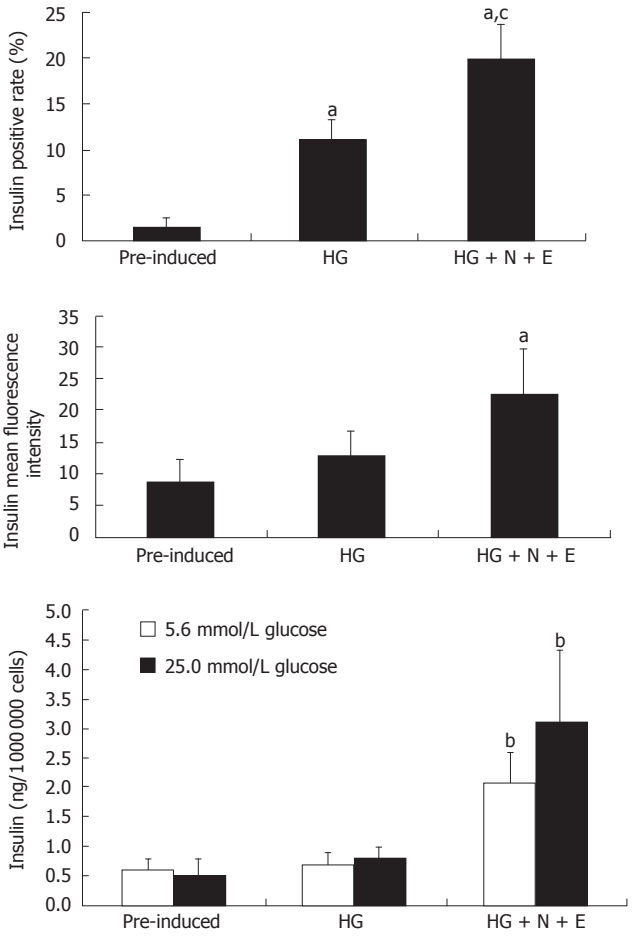

Figure 4 Insulin content and release in response to glucose stimulation of BM-MSCs in different groups. Insulin positive rate of D-MSCs was up to around 19.8% and mean fluorescence intensity was increased by 2.6 folds, which was significantly higher than that of pre-induced BM-MSCs. D-MSCs secreted a small amount of insulin into medium and the insulin was increased by 1.5 folds under the glucose challenge. $^{a}P < 0.05$ *vs* pre-induced BM-MSCs; $^{c}P < 0.05$ *vs* HG; $^{b}P < 0.01$ *vs* other group. N: nicotinamide; E: exendin-4.

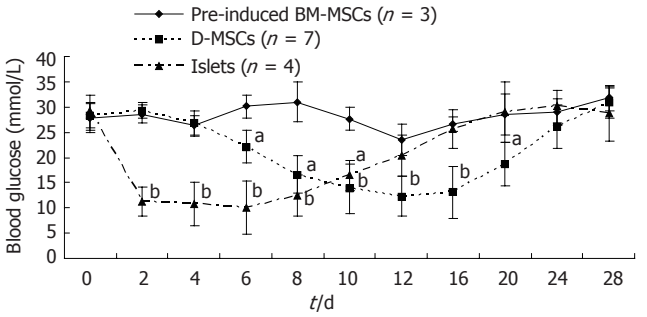

Figure 5 Glucose change of STZ-induced diabetic rats after D-MSCs transplantation *via* portal vein. Glucose levels in the D-MSCs implanted rats began to decrease at d 6 following transplantation, kept below 15 mmol/L during d 12 to d 16, and then elevated again after d 20. In contrast, glucose levels in islets implanted rats decreased at d 2, kept below 15mmol/L during d 4 to d 8, and then elevated again after d 10. Glucose levels in the pre-induced BM-MSCs implanted rats remained elevated. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* pre-induced BM-MSCs implanted rats.

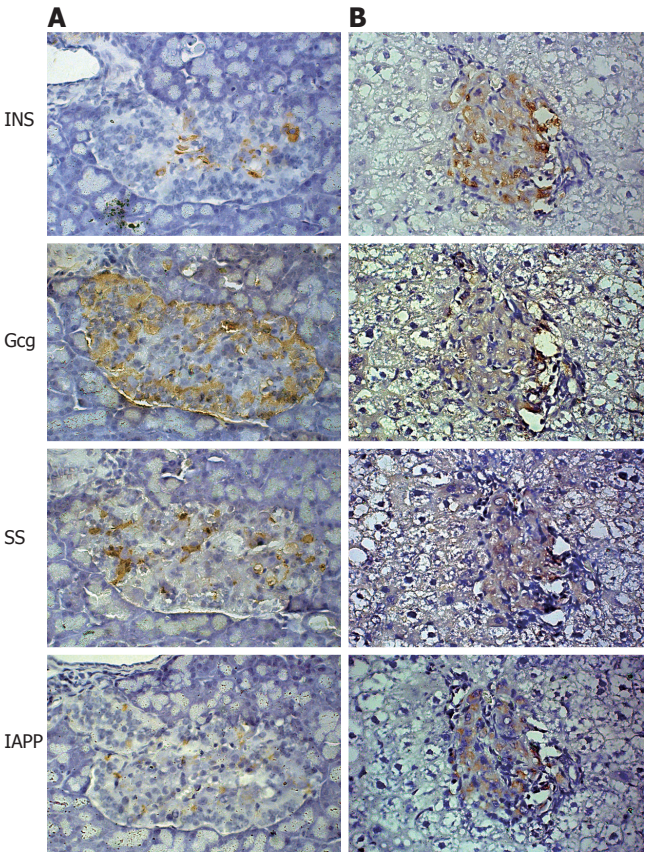

Figure 6 Islet hormones expression in the pancreas and liver of STZ-induced diabetic rat after D-MSCs transplantation via portal vein. D-MSCs grafts were located in the liver (**B**, × 400), expressing insulin (INS), glucagons (Gcg), somatostatin (SS) and islet amyloid polypeptide(IAPP); In the remaining pancreatic islets, a few insulin-expressing cells located in the center, rich Gcg-expressing cells setting at the periphery, a few SS-expressing cells and IAPP-expressing cells scattering in the islets (**A**, × 400).

doubling time is 48-72 h, and cells could be expanded in culture for more than 60 doublings[20]. Autologous transplantation of functional cells differentiated from BM-MSCs would not cause any rejection. Several *in vitro* studies have shown that bone marrow-derived stem cells are capable of being reprogrammed to become functional insulin-producing cells[9-11,21-23]. Their inducing processes are to initiate PDX-1 gene expression directly in BM-MSCs or *via* nestin-positive cells, using factors such as nicotinamide, glucose, β-mercaptoethanol, dimethyl sulphoxide, trichostatin A and so on.

We attempted to induce BM-MSCs into islet-like cells by high glucose, nicotinamide and exendin-4 (GLP-1 agonist) which were considered as potent inducers for pancreatic islet differentiation. Glucose is a growth factor for β-cells. It promotes β-cell replication *in vitro* and in vivo at a 20-30 mmol/L concentration, induces adult hepatic stem cells into pancreatic endocrine hormone-producing cells at a 23 mmol/L concentration and increases insulin content in cell lines derived from embryonic stem cells at a 5-mmol/L concentration[7,24,25]. Nicotinamide is a poly (ADP-ribose) synthetase inhibitor and could induce liver stem cells or pancreatic progenitor cells into insulin producing cells[7,8]. Whereas exendin-4 could also stimulate both β-cell replication and neogenesis from ductal progenitor cells, and inhibit apoptosis of β-cell[26,27].

Our data showed that CD45-negative and CD90/CD29 positive BM-MSCs could be manipulated toward a pathway of pancreatic endocrine cell lineages differentiation under low serum HG-DMEM *via* a still unclear mechanism. These premature cells could express insulin (Ⅰ and Ⅱ), Gcg, SS, IAPP, GLP-1R, PDX-1, Ngn3, NeuroD1, PAX-6, GLUT-2 genes, and a relatively low level of insulin, Gcg, SS and IAPP proteins. However, they showed low insulin secretion and weak glucose response. At this stage, GLP-1 receptor gene was also expressed. Nicotinamide and exendin-4, D-MSCs displayed Nkx2.2, GK and aforementioned genes and proteins, the insulin protein levels were markedly increased. Moreover, D-MSCs could secrete a small amount of insulin and show glucose response to some extent. Although a combination of nicotinamide and exendin-4 effectively promotes further differentiation of BM-MSCs in our experimental system, insulin positive rate of D-MSCs was only around 19.8%, the insulin production and glucose response were still quite lower when compared with pancreatic islets.

In order to test the function of D-MSCs *in vivo*, we transplanted the differentiated cells in STZ-induced diabetic rats *via* portal vein. At first, we established the allogeneic islets transplantation system *via* portal vein. We found that islet grafts could reduce the hyperglycemia of diabetic rats for 6-8 d after transplantation and then lost their function gradually due to allograft rejection. However, blood glucose levels of D-MSCs implanted rats began to decrease at d 6 after transplantation and kept below 15 mmol/L during d 12 to d 16, suggesting that immature D-MSCs needed further differentiation *in vivo* to display their functions. Immunohistochemical analysis revealed that D-MSCs grafts could survive in the liver of the recipient and express insulin, Gcg, SS and IAPP. Some data have suggested that transplanted stem cells derived from bone marrow could initiate endogenous pancreatic β cell regeneration and then reduce hyperglycemia in mice with STZ-induced pancreatic damage[28]. We also detected the islet hormones expression of pancreas in the recipient. Very few native islets could be found in the pancreas with only few insulin-expressing cells located in the center, just like the STZ-induced pancreatic damage, indicating that D-MSCs did not promote endogenous β cells regeneration in our experiment. After d 20, glucose levels elevated again. D-MSCs grafts lost their functions and displayed lymphocyte infiltration and apoptosis (Data not shown), which might be due to allograft rejection.

Taken together, present studies demonstrate that rat BM-MSCs may be trans-differentiated into islet-like cells *in vitro*. Portal vein transplantation of islet-like cells may alleviate the hyperglycemia of diabetic rats. These insulin-producing cells may be a potential source for antilogous transplantation without immune rejection. However, because of the transdifferentiation and dedifferentiation potency of BM-MSCs, there are many questions that remain unresolved, such as how to manipulate the differentiation process (e.g. exogenous factors, and timing of factor addition), how to push these cells to become mature β cells, will the autoantibodies responding to β-cell antigens recognize and destroy the newly generated insulin-producing cells obtained from BM-MSCs. Further research is required to address these important questions.

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RAPID COMMUNICATION

Planned second-look laparoscopy in the management of acute mesenteric ischemia

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Abstract

AIM: To investigate the role of second-look laparoscopy in patients with acute mesenteric ischemia (AMI).

METHODS: Between January 2000 and November 2005, 71 patients were operated for the treatment of AMI. The indications for a second-look were low flow state, bowel resection and anastomosis or mesenteric thromboembolectomy performed during the first operation. Regardless of the clinical course of patients, the second-look laparoscopic examination was performed 72 h post-operatively at the bed side in the ICU or operating room.

RESULTS: The average time of admission to the hospital after the initiation of symptoms was 3 d (range, 5 h-9 d). In 14 patients, laparotomy was performed. In 11 patients, small and/or large bowel necrosis was detected and initial resection and anastomosis were conducted. A low flow state was observed in two patients and superior mesenteric artery thromboembolectomy with small bowel resection was performed in one patient. In 13 patients, a second-look laparoscopic examination revealed normal bowel viability, but in one patient, intestinal necrosis was detected. In two of the patients, a third operation was necessary to correct anastomotic leakage. The overall complication rate was 42.8%, and in-hospital mortality rate was 57.1% (n = 6).

CONCLUSION: Second-look laparoscopy is a minimally invasive, technically simple procedure that is performed for diagnostic as well as therapeutic purposes. The simplicity and ease of this method may encourage wider application to benefit more patients. However, the timing of a second-look procedure is unclear particularly in a patient with anastomosis.

INTRODUCTION

Acute mesenteric ischemia (AMI) resulting in intestinal ischemia or infarction is associated with an extremely serious prognosis and mortality rate ranging from 40%-100%[1-3]. Acute mesenteric vascular ischemic diseases are diagnosed more commonly as a consequence of the aging population and often result in emergency bowel resection. Abdominal second-look may occasionally be necessary in cases of doubtful bowel viability or intra-abdominal sepsis after primary anastomosis[4,5]. In 1965, Shaw[6] introduced the "second-look laparotomy" to overcome the difficulty in assessing the adequacy of bowel resection during surgery.

Second-look entails early surgical re-exploration to check the viability of intestinal loops and is the mainstay of AMI surgical treatment[7,8]. When a second-look surgery is indicated, second-look laparoscopy may be a useful alternative to conventional surgery, because it prevents critically ill patients from the trauma and risks of re-laparotomy and can be performed as a bed-side operation in the intensive care unit[4]. In this study, we aimed to determine the outcome of patients with AMI with or without bowel necrosis, who were subjected to a second-look laparoscopy.

MATERIALS AND METHODS

Between January 2000 and November 2005, 71 patients were operated to treat acute mesenteric ischemia (AMI) at Istanbul University, Istanbul Faculty of Medicine, Trauma and Emergency Surgery Service. Triple-contrast computed tomography (CT) scanning or CT angiography was used to confirm either arterial occlusion or bowel changes compatible with AMI. Fifty-seven patients were excluded and did not undergo a second-look laparoscopy because the bowel resection required an ostomy during the first procedure. The remaining 14 patients underwent a second-

Table 1 Clinical characteristics and outcome of the fourteen patients

No.	Comorbid disease	Duration of symptom onset before admission to hospital	Duration of hospital stay (d)	Results of second-look	Result
1	-	4 d	13	Normal	
2	IHD	7 d	13	Normal	
3	IHD + HT	5 h	52	Normal	Died
4	-	1 h	23	Normal	Died
5	Acute pancreatitis, IHD, HTN	1 h	18	Normal	Died
6	IHD	8 h	10	Normal	
7	Epilepsy	3 d	10	Normal	
8	DM + HTN + AF	10 h	10	Normal	
9	HTN+AF	2 d	10	Normal	
10	DM + HTN + IHD	7 d	24	Normal	Died
11	DM	7 d	8	Normal	
12	-	9 d	38	Partial small intestine resection	Died
13	DM + IHD + HTN	2 d	16	Normal	
14	DM + HTN + AF + Toxic goiter + Asthma	3 d	17	Normal	Died

IHD: Ischemic heart disease; HTN: Hypertension; DM: Diabetes mellitus; AF: Atrial fibrilation.

look laparoscopic examination. In this study, we only discuss those 14 patients, who underwent a second-look laparoscopy.

In our clinic, our policy is to perform a second-look laparoscopy for all patients operated on for AMI. Regardless of the clinical course of patients during the first operation when bowel viability was suspected and a low flow state was detected or bowel resection and anastomosis were performed, we performed a second-look laparoscopy within 72 h following the first operation at the bed side in the ICU or operating room. At the end of the operation, a 10-mm laparoscopic trocar was inserted into the left lower quadrant of the abdomen prior to closing the abdominal wall. Data were collected on patients' demographics, co-morbid diseases, clinical signs and symptoms, intra-operative findings and hospital course.

All patients were given a low molecular weight heparin (Enoxaparin sodium-Clexane®, 1 mg/kg per day) treatment once AMI was diagnosed and continued on enoxaparin until the patient received an oral anticoagulant (Warfarin-Na), if indicated. When the patient was stabilized, an echocardiography was performed. Mortality was defined as in-hospital death. The study was approved by the Institutional Review Board.

RESULTS

There were nine men and five women with a median age of 68 years (range, 45-76 years). The median hospital stay was 16 d (range, 1-52 d). The most common co-morbid diseases were hypertension (HTN) in 7 (50%) patients (Table 1). Abdominal pain was present in all of the patients. Nausea was the second most frequent symptom and observed in 10 (71.4%) patients, followed by vomiting in 7 (50%) patients, and bloody diarrhea in 3 (21.4%) patients. The median time of admission after the onset of symptoms was 3 d (range, 5 h-9 d).

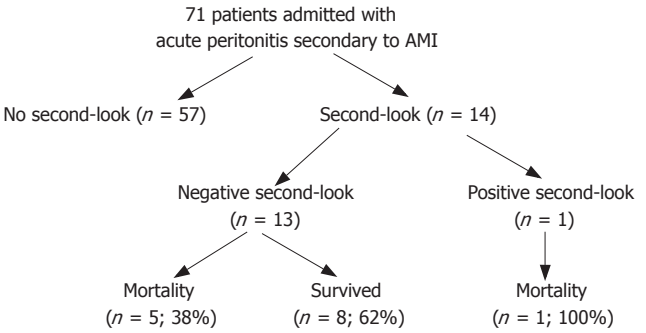

Figure 1 Outcome of patients with acute mesenteric ischemia treated with a second-look procedure.

In 11 patients, small and/or large bowel necrosis was detected. Bowel resection and primary anastomosis were performed during the first procedure. In two patients, non-occlusive mesenteric ischemia (NOMI) without bowel necrosis was detected. In one patient, who was admitted to the emergency service within 3 h following abdominal pain, superior mesenteric artery (SMA) thromboembolectomy was performed because of an embolism in the SMA.

In 13 patients, a second-look laparoscopic examination showed normal viable intestinal loops and a normal healing anastomosis (Figure 1). In one patient, who previously underwent SMA thromboembolectomy, intestinal necrosis was found and spanned the distance from 70 cm distal to the ligament of Treitz to 10 cm proximal to the ileocecal valve, and therefore a partial small bowel resection with end jejunostomy and end ileostomy were performed. In two of the patients, the third operation was required due to peritonitis and leucocytosis, an anastomotic leakage was found in both patients on 6th post-operative day; so ileostomy or colostomy was performed.

Overall in-hospital mortality was reported in 6 (57.1%) patients. Multiorgan failure caused death in 4 (66.6%) patients, being the most common cause of mortality. One patient died secondary to myocardial infarction and one died from sepsis. The overall complication rate was 42.8%. Peptic ulcer perforation occurred in one patient, who had previously suffered from acute pancreatitis, despite H_2 blocker prophylaxis. This patient was re-operated for pancreatic necrosis. An another patient had wound dehiscence.

DISCUSSION

The mortality associated with AMI decreased from 80%-90% in the 1970's to 60%-70% in the 1980's and 1990's[2,3]. This has been attributed to earlier diagnosis secondary to increased awareness, aggressive angiography, surgical and non-surgical blood flow restoration, resection of all necrotic bowel, second-look laparotomy or second-look laparoscopy and supportive intensive care[8,9].

Second-look laparotomy remains the gold standard for determination of further bowel viability and an operation is the only way to remove dead bowel. During the operation, bowel viability can be assessed by physical examination (inspection of bowel and palpation of vessels), hand-held Doppler ultrasound examination and intravenous injection of fluorescein[9-11]. These techniques are helpful but far from being sensitive and specific enough to allow omitting the second-look procedure[10-13]. Indications for the second-look procedure remained viable even when more objective methods such as Doppler ultrasonography and fluorescein testing became available. We use neither Doppler nor flurescein testing pre-operatively. We believe that if the bleeding is enough on the cutting end and the arterial pulse is palpable on the mesenteric side of the bowel in a normotensive patient, the patient is amenable to anastomosis, unless intra-abdominal sepsis or peritonitis is present.

In critically ill patients, conventional laparotomy is associated with certain general and access-related risks[5]. Second-look laparoscopy has become a diagnostic technique with potential therapeutic options. Second-look procedure has become more common in mesenteric vessel occlusion with uncertain intestinal viability observed during the primary surgery[1-3]. This procedure can also be applied under local anesthesia and be performed in the intensive care unit under sedation or analgesia. We prefer to perform the second-look procedure in the operating room, unless the patient is hemodynamically unstable.

In a large French study, although the overall survival of patients with AMI improved from the early 1980's to early 1990's, the percentage of second-look procedures remained unchanged[1]. Endean *et al*[16] stated that 15 of 43 (35%) patients with AMI with either thrombosis or embolism underwent a second-look procedure. In our clinic, a second-look laparoscopy is warranted in patients with AMI, if they exhibit either a low flow state, or have had a bowel resection with anastomosis during the first operation. However, among 71 patients, only 14 patients underwent a second-look laparoscopy. The reason for the lower incidence of a second-look laparoscopy was that most patients were transferred to our clinic from other hospitals. This delay in diagnosis led to bowel perforation and peritonitis resulting in the creation of a stoma and the abdomen was closed by using a Bogota bag.

Second-look laparoscopy is a safe method that decreases the negative second-look laparotomy risk in critically ill patients[4]. In reviewing results on 92 patients, Levy *et al*[17] stressed the beneficial role of a second-look on patient survival, although only 14% of their patients were exposed to this procedure. Since there are no predictive criteria for the progression of ischemia and not all patients undergo second-look procedures, some patients will undergo unnecessary surgical and anesthesiological procedures (negative second-look), and others in whom a surgical second-look might be beneficial, will not be operated on[7]. At this critical point when bowel ischemia is suspected, a laparoscopic second-look is important, because it can reduce severe unnecessary anesthesiological and surgical trauma to the patients by easily replacing the open surgical procedure for permanent treatment.

Although studies have advocated that laparoscopic second-look is a routine substitute of surgical second-look, there exist controversies regarding the timing of second-look operations. Practically, re-operation may be performed within 24 h. However, we prefer to perform the second-look operation within 72 h, which promotes bowel viability and anastomotic healing. We performed a second-look laparoscopy for our 14 patients during the ensuing 72 h. To our knowledge, the majority of anastomotic leakage occurs at 3rd to 5th post-operative days. We believe this contributes to early detection of leakage and prevent peritonitis. Although the laparoscopic findings were normal, we found anastomostic leakages in two patients at 6th post-operative day.

Denecke and Stiegler[18] stated that they performed second-look to control viability or lavage in 36 of 87 AMI patients. In 10 of 87 patients, a resection was performed. Unfortunately, only five of these patients survived. By the second-look procedure, 5 of 87 patients could be saved[18]. However, even in earlier studies that reported an advantage of the second-look procedure, the best survival rates were not much higher than 65%[16,19].

Anadol *et al*[20] compared open and laparoscopic second-look procedures in AMI patients. In the first group of 41 patients, the abdomen was closed after the first procedure. In the second group of 36 patients, a 10-mm trocar was inserted before closing the abdomen and a second-look intervention was performed by telescope in 23 patients. Seventy percent of re-laparotomies revealed nothing and were unnecessary. Eight percent of the re-laparoscopy group required re-resection while 87% of patients were rescued from unnecessary laparotomies[22].

Finally, a second-look laparoscopy is minimally invasive and technically simple. Laparoscopy has a shorter operative time compared to conventional laparotomy. It can not only be performed as a bedside procedure and sometimes without anesthesia, but also minimizes the risks of a redo-laparotomy.

The simplicity and ease of this method may encourage wider application to benefit more patients. However, prospective randomized studies are required for clarification if second-look procedures make a difference in outcomes.

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RAPID COMMUNICATION

Frequent loss of heterozygosity at 8p22 chromosomal region in diffuse type of gastric cancer

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Abstract

AIM: To study the loss of heterozygosity (LOH) at 8p21-23 locus in diffuse gastric cancer.

METHODS: To evaluate the involvement of this region in gastric cancer, we used eight microsatellite markers covering two Mb of mentioned region, to perform a high-resolution analysis of allele loss in 42 cases of late diffuse gastric adenocarcinoma.

RESULTS: Six of these STS makers: D8S1149, D8S1645, D8S1643, D8S1508, D8S1591, and D8S1145 showed 36%, 28%, 37%, 41%, 44% and 53% LOH, respectively.

CONCLUSION: A critical region of loss, close to the *NAT2* locus and relatively far from *FEZ1* gene currently postulated as tumor suppressor gene in this region.

Key words: Loss of heterozygosity; Tumor suppressor genes; diffuse type of gastric cancer; STS marker; *N*-Acetyltransferase 2; *Fez1*

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INTRODUCTION

Adenocarcinoma of the stomach (ACS) is the second most common cancer worldwide and there are two distinct biological and etiological subtypes of ACS: (*a*) the intestinal and (*b*) the diffuse-infiltrative type. The individual's risk of the intestinal disease is dominant in countries with a high incident of gastric cancer. Although its incidence is decreasing, ACS is the second leading cause of cancer mortality in many countries[1,2].

A recent cancer survey by the Iranian Ministry of Health and Medical Education (IMHME) revealed that gastric adenocarcinoma is the most common fatal cancer in Iran, with a wide variation of death rate among different provinces. According to the recent cancer statistics, deaths due to gastric cancer constitute about 39% of all deaths due to cancer each year in some parts of Iran[3].

Diet and environment are important factors in the intestinal form of ACS, which is associated with chronic atrophic gastritis and intestinal metaplasia of the gastric mucosa. In addition, environmental factors have influence on incidence of the diffuse (i.e. infiltrative) form of ACS[4]. One of the strong tools to genetic analyses is loss of heterozygosity (LOH) that consequently leads to loss of function of tumor suppressor genes. Inactivation of first normal allele mainly occurs by point mutation, followed by deletion or loss of second allele[5]. Hence, to LOH analyzing in a region, usually the microsatellite STS markers are used. These markers enable to trail contemporary two alleles of a gene[6].

Frequent LOH at specific chromosomal regions in certain tumors implies the presence of suppressor genes. Recent allelotyping studies have shown that allelic losses on the short arm of chromosome 8, particularly at bands 21-23.1, are frequently associated with various tumors, including prostate cancer[7,8], breast cancer[9,10], head and neck squamous cell carcinoma[11,12], urinary bladder carcinoma[13,14], hepatocellular carcinoma[15], lung cancer[16] and colorectal cancer[17]. Additionally, frequent deletion at 8p22 has been shown strongly associated with gastric cancer progression[18]. These observations suggest that chromosomal region 8p21-23.1 plays a critical role in the development of various tumors.

Experimentally functional evidence by chromosome transfer into tumor cells at 8p region showed the presence of one or more putative tumor suppressor gene(s) in this region[19]. In addition, micro-cell fusion experiments suggested the possible location of metastasis suppressor

gene(s) at 8p[20, 21]. It is possible that at the locus 8p, two or more genes will be involved in suppressing of cancer development. Efforts toward positional cloning of the suppressor gene(s) allowed the isolation of an important candidate tumor suppressor *FEZ1* gene at 8p22. However, the significance of *FEZ1* in the tumor-development or progression remains confused[22-24].

Accordingly, in this first study from Iran, eight microsatellite STS markers were selected to analyze frequency of allelic loss in 42 cases of late diffuse type of gastric cancer.

MATERIALS AND METHODS

Subject

Paraffin embedded tissues of 42 patients were analyzed with advanced locally diffuse type of gastric cancer registered in RCGLD registry system from 2003 to 2005.

DNA Extraction: Formalin-fixed, paraffin-embedded tissue blocks were sectioned with 5 μm thickness. They stained (Hematoxylin and Eosin), and viewed to confirm histological grading. Using the stained dissected slides as templates, two 20-μm section fragment of paraffin-embedded tissue were placed in two sterile tubes as source of tumoral and normal samples, separately. Deparaffinization was performed with Xylene, followed by DNA extraction as described previously[25].

Microsatellite STS marker selection

LOH analysis by paired normal-tumor microsatellite PCR was performed using eight well-mapped microsatellite markers (Table 1). These markers cover approximately two Mb of 8p22 region. Figure 1 shows the chromosomal positions of the selected markers, based on the sequence tagged site database (http://www.ncbi.nlm.nih.gov), with supplementary mapping information, provided through the Cooperative Human Linkage Center database (http://www.chlc.org), the Genome Database (http://www.gdb.org), the Genetic Location Database (http://www.cedar.genetics.soton.ac.uk/public_html).

PCR of STS microsatellite markers

In a total volume of 25 μL the PCR contained 2 μL DNA sample solution, 200 μm of all four deoxynucleotide triphosphates (dNTP), 50 pmol of each forward and reverse primers, 0.2 μL super Taq polymerase (Roche), 2 μL of 10X PCR buffer (Roche), and 3 mmol/L $MgCl_2$ (Roche). The following thermal cycling conditions were employed for all reactions: an initial denaturation step of 5 min, followed by 35 cycles of denaturing, annealing, and extension (30 s each) and a final 20-min extension step. A denaturing temperature of 95℃ and an extension temperature of 72℃ were used and annealing temperatures for the different primer sets were optimized as necessary (Table 1)

Gel electrophoresis, staining and LOH analyzing

PCR-products were size-separated on a Biorad 165-3860 Sequi-gene using 5 μL of the sample was then loaded onto a 60 g/L polyacrylamide gel containing 7 mol/L urea, 450 mmol/L Tris-borate (pH 7.5) and 1 mmol/L EDTA (pH 7.0) running buffer. Loaded gels were electrophoresed for 2-4 h (depend on PCR product size bands) and stained with $AGNO_3$ method[26].

Because of low quality of specimens, 17 of 42 samples were excluded and the remains normal and tumor paired-samples from 25 late diffuse type of gastric cancer were screened for LOH at 8p22. LOH analysis was performed as described previously[27,28]. Each locus scored for LOH according to the absence (allelic loss) or the disequilibrium (allelic imbalance) of signal from one allele in the tumor-DNA-amplification product as compared with the normal one. Reduction of > 50% of band intensity was considered to loss. (Figure 2)

RESULTS

Allelic loss for at least one locus detected in 76% (19/25) cases examined. Eight loci were tested in all of the matched normal and tumor samples (Table 1). Two of these STS markers (D8S1948, D8S280) have been excluded because of non-informatively. In other markers allelic loss ranged were 36% (4/11) for D8S1949, 28% (5/18) for D8S1645, 37% (7/19) for D8S1643, 41% (5/12) for D8S1508, 44% (4/9) for D8S1591 and 53% (7/13) for D8S1145. Allelic imbalance for at least one locus found in 76% of tumor samples. Figure 2 shows the pattern of allelic loss for each case. In eight cases, loss of one allele tends to telomeric and in four cases to centromeric region. Other cases showed loss in the middle point of 8p22 region.

DISCUSSION

In this study we examined a region of chromosome 8p favored as potentially harboring tumor suppressor genes[7-18]. The allelotyping performed in a region less than two Mb at 8p22 to identify a common deletion in the late diffuse type of gastric carcinoma. Allelic imbalance for at least one locus found in 76% of tumor samples. However, the diffuse-type of gastric cancer has a higher normal cell content and the DNA impurities maybe superimposed to infrequent chromosomal losses[28]. Therefore, the frequency of 8p22 deletion in this study is considerable. The unique connection of this locus in the carcinogenesis introduces two possibilities: (1) 8p22 locus is one of phenotype-determined events tends to develop diffuse gastric cancer; (2) or alternatively, 8p22 locus participate in late stage of diffuse gastric cancer and is more likely to harbor chromosome instabilities. Furthermore, our data allowed us to define a minimal region of allelic loss at 8p22 to a segment around D8S1145 marker with a LOH rate of 53% (Figure 2); make it a good candidate to harbor putative TSG.

Several candidate cancer-susceptibility genes at 8p22, such leucine zipper tumor suppressor 1 (*LZTS1*) or *FEZ1*[22-24], deleted in liver cancer *DLC1*[29] and mitochondrial tumor suppressor gene1 *MTUS1*[30] are other candidate TSGs in this region. Nevertheless, the minimal region of loss in our tumor samples was telomeric

Table 1 Markers and their characteristics

Locus	Forward primer (5'-3')	Reverse primer (5'-3')	Size range (bp)	Annealing temperature (℃)	(%) Informative	(%) LOH
D8S1948	TTACAAAACATACCCAGTGTTTGG	CTTTTTAGTGCTTGAGACTGTCTCC	110-111	58	< 10	Exclude
D8S1949	TGTCTTACAGCTCTCCCTCTCC	CAGTAAGGATCACCAAGACAAGG	106-107	65	40	36
D8S1645	GTTCAACTGTTGATTTTTTGACAA	CTTTTTAGTTAATTCCCATCAGCA	176-177	62	72	28
D8S1643	AGGCCTTGTAAGTGATAAAGGC	TTCTCCATCAACCTTTTGGC	100-101	64	80	37
D8S280	CAATTTCATTGCTAGGTGTATATCC	CTGTTTTATGGCTGAATAGTGTCC	224-232	59.5	< 10	Exclude
D8S1508	AAAATTCCTACCTTGCTATGAACA	CTGCACGTAACTCTCCACCA	181-182	61.5	50	41
D8S1591	CAAAGATTTCTTTTTATTCACCTGC	TTTCTTTAGATGGAGTCCATTGC	208-209	59.5	36	44
D8S1145	TGCTAACTGGCACGGTCAC	CAATCCCAGTAATCTATAACTTCA	261-289	63	56	53

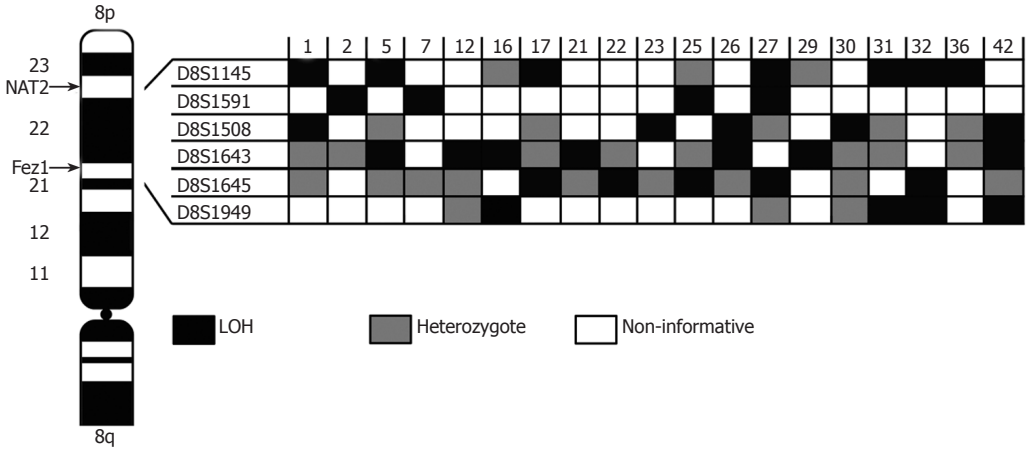

Figure 1 LOH pattern of markers and chromosomal positions. Case number is illustrating above each column. Microsatellite STS markers listed in order from telomere to centromere together with their genetic location. *Fez1* gene locus is close to D8S1949 marker with 36% LOH and *NAT2* gene locus is close to D8S1145 marker with 53% LOH, respectively.

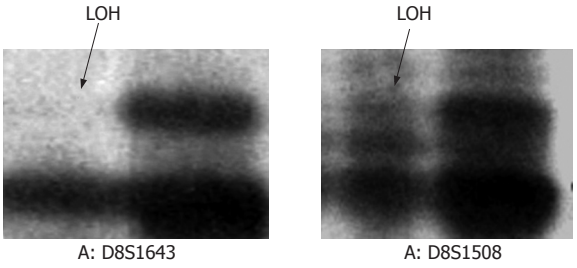

Figure 2 Showed LOH in two markers.

to these genes and the D8S1145 marker with the highest LOH rate is close to the *NAT2* locus.

The *N*-acetyltransferase isoezymes, *N*-acetyltransferase 1 (*NAT1*) and *N*-acetyltransferase 2 (*NAT2*) catalyze either *N*-acetylation of aromatic amine and hydrazine drugs or *O*-acetylation of *N*-hydroxy-aromatic and heterocyclic amines, and have a primary role in the activation and/or deactivation of a large and diverse number of environmental pollutants[31]. Because *NATs* activate and/or deactivate environmental pollutants, some of which have been implicated in the etiology of cancers, it has been suggested some polymorphisms that alter the function of *NAT* genes may be risk factors for the disease[32]. It is often suggested that human *NAT2* activity is highest in the liver and gastrointestinal tract[33]. Certain polymorphisms are associated with a decrease in *N*-acetyltransferase2 activity leading to a possible increased risk factor to develop bladder, gastric, lung and prostate cancers[32-35]. In other hand, occurring of diffuse gastric cancer in proximal gastric tissue is abundance than other section of gastric tissue[36,37] and *NAT2* only expressed in this region of stomach[38,39].

Furthermore, accumulating evidences indicate that both genetic and epigenetic changes associate with diffuse gastric cancers[23,40]. Ethnic background suggested being associated with differences in disease aggression and outcome in Asian populations[41,42]. Based on mentioned reports and our LOH rate around *NAT2* locus, we hypothesize that the loss of *NAT2* gene might influences the progression of this form of cancer or this region harbored another tumor suppressor gene far from of *FEZ1* locus.

Further studies will define the key gene targets of alteration on 8p22-23.1 in gastric cancers.

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RAPID COMMUNICATION

Phase 1 human trial of autologous bone marrow-hematopoietic stem cell transplantation in patients with decompensated cirrhosis

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Abstract

AIM: To evaluate safety and feasibility of autologous bone marrow-enriched CD34$^+$ hematopoietic stem cell Tx through the hepatic artery in patients with decompensated cirrhosis.

METHODS: Four patients with decompensated cirrhosis were included. Approximately 200 mL of the bone marrow of the patients was aspirated, and CD34$^+$ stem cells were selected. Between 3 to 10 million CD34$^+$ cells were isolated. The cells were slowly infused through the hepatic artery of the patients.

RESULTS: Patient 1 showed marginal improvement in serum albumin and no significant changes in other test results. In patient 2 prothrombin time was decreased; however, her total bilirubin, serum creatinine, and Model of End-Stage Liver Disease (MELD) score worsened at the end of follow up. In patient 3 there was improvement in serum albumin, porthrombin time (PT), and MELD score. Patient 4 developed radiocontrast nephropathy after the procedure, and progressed to type 1 hepatorenal syndrome and died of liver failure a few days later. Because of the major side effects seen in the last patient, the trial was prematurely stopped.

CONCLUSION: Infusion of CD34$^+$ stem cells through the hepatic artery is not safe in decompensated cirrhosis. Radiocontrast nephropathy and hepatorenal syndrome could be major side effects. However, this study does not preclude infusion of CD34$^+$ stem cells through other routes.

Key words: Cirrhosis; Bone marrow; Stem cell; Transplantation; Quality of life; Model of End-Stage Liver Disease score

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INTRODUCTION

Cirrhosis represents a late stage of progressive hepatic fibrosis characterized by distortion of the hepatic architecture and the formation of regenerative nodules. Orthotopic liver transplantation (OLT) is the standard treatment modality in patients with decompensated cirrhosis. However, it has several limitations such as shortage of organ donors, high cost, and several complications.

For example, in Iran the minimum number of patients who need liver transplant each year is around 1000[1], but the maximum number of OLT is only 100 per year[2]; therefore, the majority of patients with end stage liver disease in Iran are presently dying at the end of their natural history of liver disease. Living donor liver transplantation provides one means to expand organ availability. However, there is a real need for an alternative therapies for end stage liver disease.

Preliminary experience with clinical hepatocyte transplantation during the past decade has provided proof of concept that cell therapy can be effective for the treatment of some liver diseases. Recent progress in cell biology resulting in the isolation and characterization of bone marrow stem cells and progenitor cells further increases the expectation for a new approach to the treatment of genetic and chronic liver disease[3].

There are at least two types of stem cells in the human

bone marrow; mesenchymal stem cells, and hematopoietic stem cells (HSCs). HSCs are $CD34^+$ and $CD133^+$ and they can give rise to all lineages of blood cell differentiation. Recently, intracoronary infusion of bone marrow stem cells was reported to be safe and effective in patients with acute myocardial infarction[4].

Furthermore, *in vivo* trans-differentiation of human HSCs to functional hepatocytes has been demonstrated[5]. Also, it has been shown that infusion of bone marrow stem cells to animal models of liver cirrhosis can lead to regression of liver fibrosis[6]. Recently, am Esch *et al*[7] reported that portal administration of autologous $CD133^+$ HSCs accelerated liver regeneration. We hypothesized that infusion of HSCs may help to reverse liver failure in patients with decompensated cirrhosis. Thus, we conducted a phase 1 human trial to evaluate safety and feasibility of autologous bone marrow-enriched $CD34^+$ HSC transplantation in patients with decompensated cirrhosis.

MATERIALS AND METHODS

Preparation of bone marrow-enriched $CD34^+$ cells

One day before stem cell infusion, a total of 200 mL of bone marrow was aspirated from four different sites of the iliac crest in the right and left side (50 mL at each site) of the patients in a standard fashion. The harvested bone marrow was placed in sterile tubes containing 1500 U/50 mL of heparin sulfate to avoid platelet clumping. The procedure of stem cell isolation was performed in a clean room (FS 209 E & ISO 14644). To reduce the volume of red blood cells, hydroxyethyl starch was used.

Mononuclear cells were separated by Ficoll-Hypaque (Lymphodex, inno TRAin, H9L6114) and then these cells were diluted in cliniMACS buffer. The bone marrow LD-MNCs were incubated for 45 m at 4℃ with the CD34 monoclonal antibody (mAb) directly labeled to microbeads (MACS, Miltenyi Biotec GmbH, 171-01, Bergisch Gladbach, Germany), washed with cliniMACS buffer and placed on a column in the miniMACS cell separator (Miltenyi Biotec). The labeled cells were separated using a high-gradient magnetic field, and eluted from the column after their removal from the magnet. The positive fraction was then placed on a new column and the magnetic separation step repeated. At the end of the separation, the cells were counted and assessed for viability using Trypan Blue dye exclusion; their purity was determined using a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA, USA). Enriched $CD34^+$ cells were stored at 4℃ in 2% human serum albumin (Human Albumin 20%, USP, Bayer, 683-20) in a sterile tube until the stem cell infusion the next day.

Transplantation of HSCs

After local anesthesia, puncture of right femoral artery was performed, and 5 French sheaths were inserted. Simon Ⅲ catheter advanced to the descending aorta, and catheterization of celiac axis and then hepatic artery was performed. The mean duration of catheterization was 9.5 m (range: 5-15 m). Nonionic low osmolal radiocontrast agent was used to visualize the hepatic artery. Then $CD34^+$ stem cells were selectively applied to the hepatic artery as equal aliquots of 10 mL, taking an average time of 10 m. After that, the catheter was flushed with 10 cc of normal saline and the procedure was finished. After the stem cell infusion the catheter and the sheath were removed.

Patients

The proposal was designed to include 6 patients with decompensated cirrhosis. The project was approved by the Ethics Committee and the research council of digestive disease research center, Tehran University of medical sciences. The written informed consent was assigned by the patients. Inclusion criteria were age 18-60 years; chronic liver failure, ultrasonographic evidences of cirrhosis and portal hypertension, abnormal serum albumin, and/or bilirubin and/or prothrombin time (PT); Child-Pough score of 7 or more. Exclusion criteria were history of moderate to severe hepatic encephalopathy or variceal bleeding during the last 2 mo before enrolment; serum Cr $\geqslant$ 2 mg/dL, or GFR < 40 mL/min; serum sodium < 129 meq/L; serum AST or ALT more than 3 times normal; lines of evidence of active autoimmune liver disease (serum gammaglobulin > twice normal; serum transaminases > 120 U/L); human immunodeficiency virus or hepatitis C virus seropositivity; serum hepatitis B virus DNA of more than 10 000 copies/mL in patients with positive hepatitis B surface antigen; lines of evidence of extrahepatic biliary diseases (e.g. presence of primary sclerosing cholangitis, or dilated common bile duct on ultrasonography; presence of active untreated infectious disease; presence of hepatic, portal, or splenic vein thromboses on Doppler ultrasonography; presence of severe comorbid diseases (e.g. severe renal, respiratory, or cardiac disease), or presence of any types of malignancy; history of alcohol use, or use of hepatotoxic drugs within the last 6 mo before enrolment; active substance abuse; lack of a supportive family; and unwilling to assign the inform consent. All patients were on the waiting list of liver transplantation.

Follow up visits and outcome measures

Patients were admitted and observed in Shariati hospital (Tehran, Iran) for 7 d. The following tests were performed at d 0, 1, 3, 5, and 7, wk 2, 3, and 4, mo 2, 3, and 6 post-transplantation: complete blood counts, PT, and international normalized ratio (INR), serum albumin, urea, creatinine, aspartate aminotransferase (AST), alanine aminotransferase (ALT), serum alkaline phosphatase, serum total and direct bilirubin, alfa-fetoprotein. Also, 10 mL of the patients' serum samples were collected and stored frozen at -70℃ at each visit. Liver volume of the patients was measured at baseline and at the end of follow up by multislice spiral CT scan without administration of intravenous contrast. The SF-36 questionnaire which was previously validated in Persian language[8] was completed by the patients at 1 d before the stem cells infusion, and was repeated at the end of follow up.

Primary aim of the study was to assess safety and feasibility of the study. Secondary end points were to assess changes in MELD score, liver volume, and quality of life of the patients at the end of follow up.

Table 1 Baseline characteristics of the patients

	Patient 1	Patient 2	Patient 3	Patient 4
Age (yr)	40	53	45	53
Gender	Male	Female	Male	Female
Etiology of cirrhosis	Hepatitis B	PBC	Cryptogenic	AIH
Medications	Lamivudine, Spironolactone, Furosmide	UDCA, Spironolactone, Furosmide	Spironolactone, Furosmide	Spironolactone, Furosmide

UDCA: Ursodeoxy-cholic acid; AIH: Autoimmune hepatitis; PBC: Primary biliary cirrhosis.

Table 2 Paraclinical data of the patients at baseline and at the end of follow up

	Patient 1		Patient 2		Patient 3		Patient 4	
	Baseline	mo 6	Baseline	mo 6	Baseline	mo 6	Baseline	mo 6[1]
Edema	2+	None	2+	2+	1+	1+	2+	–
Ascites	Moderate	None	Moderate	Moderate	Moderate	Moderate	Severe	–
Serum albumin (g/dL)	3.8	4.2	2.9	3.1	2.5	2.8	2.6	–
PT (seconds)	14	14.6	20.7	17	18.7	16.7	19.5	–
INR	1.2	1.3	2.2	1.8	1.9	1.6	2	–
Cr (mg/dL)	1.2	0.83	0.95	1.8	0.72	0.8	1.37	–
Total bilirubin (mg/dL)	1.2	1.87	3.31	5	2.37	2.02	19.85	–
Direct bilirubin (mg/dL)	0.4	0.43	2.03	2.8	0.66	0.82	13.82	–
AST (IU/mL)	43	52	52	75	62	37	[2]202	–
ALT (IU/mL)	31	34	23	30	30	15	[2]163	–
AFP (mcg/L)	4.7	3.7	3.48	5.5	1.7		3.8	–
MELD score	11	12	20	25	17	14	29	–
Liver volume (cm^3)	1205	1180	960	NA	896	474	1159	–

[1]Since patient 4 died two weeks after undergoing the procedure. There is no data of her follow up on mo 6; [2]Serum aminotransferases of the patient 4 was less than 3 times of upper limit of normal at screening. PT: Prothrombin time; INR: International normalized ratio; Cr: Serum creatinine; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; AFP: Alpha fetoprotein; MELD: Model for end stage liver disease. NA: not available.

Evaluation of safety and feasibility

Patients' safeties were evaluated at each visit according to the above mentioned schedule. Clinical, laboratory, and safety-related data were prospectively collected. Procedural complications were defined as any hemodynamic instability during the cell infusion. Major side effects were defined as development of any of the following complications during the follow up: acute renal failure, worsening hepatic decompensation that requires urgent liver transplantation, progressive elevation in serum AFP, or development of liver mass on follow up CT scans.

RESULTS

Viability and function of HSCs

The mean volume of bone marrow aspirated from the ilium of the patients was 215.8 mL. The mean number of mononuclear cells achieved from the patients' bone marrow was 3.13×10^8 cells. The mean number of $CD34^+$ cells achieved after isolation was 5.25×10^6 (range: 2.5-8 $\times 10^6$). The mean rate of viability of the cells was 90.75%. The mean purity of $CD34^+$ cells was 90.5%.

Clinical results

The study was designed to enroll 6 patients. Four patients (2 male, 2 female) with the mean age of 47.8 years (range: 40-53) were enrolled and underwent the procedure (Table 1).

$CD34^+$ stem cells were slowly infused through the hepatic artery. Vital signs of the patients remained stable during the stem cell infusion. The results of the study are shown in Table 2 and Figure 1.

There were no any adverse effects in patients 1, and 3 during the 6 mo of follow up. Patient 2 did not experience any side effects until mo 5. However, after that she developed some degree of renal failure. The correlation between the procedure and renal failure in this patient is unclear. Patient 4 developed progressive renal failure, and went on type 1 hepatorenal syndrome and died of liver failure prior to urgent liver transplantation. The trigger factor for acute renal failure was most probably radio-contrast nephropathy. Afterwards, the project was prematurely stopped.

DISCUSSION

Cell based therapies are increasingly studied in various types of human diseases[4,9,10]. However, safety issues should be carefully considered in these novel treatment approaches. Recently, Gaia *et al*[11] mobilized $CD34^+$ cells and observed bone marrow-derived cells may represent an easy immature cell source potentially useful for novel approaches for liver regeneration. Gordon *et al*[12], reported that HSCs infusion through the portal vein or hepatic artery was safe and may be effective in decompensated cirrhosis.

However, in our study, one patient developed some degrees of renal failure 5 mo after undergoing the procedure. Most importantly, another patient developed radiocontrast nephropathy and rapidly progressed to type 1 hepatorenal syndrome, and died of liver failure before embarking on

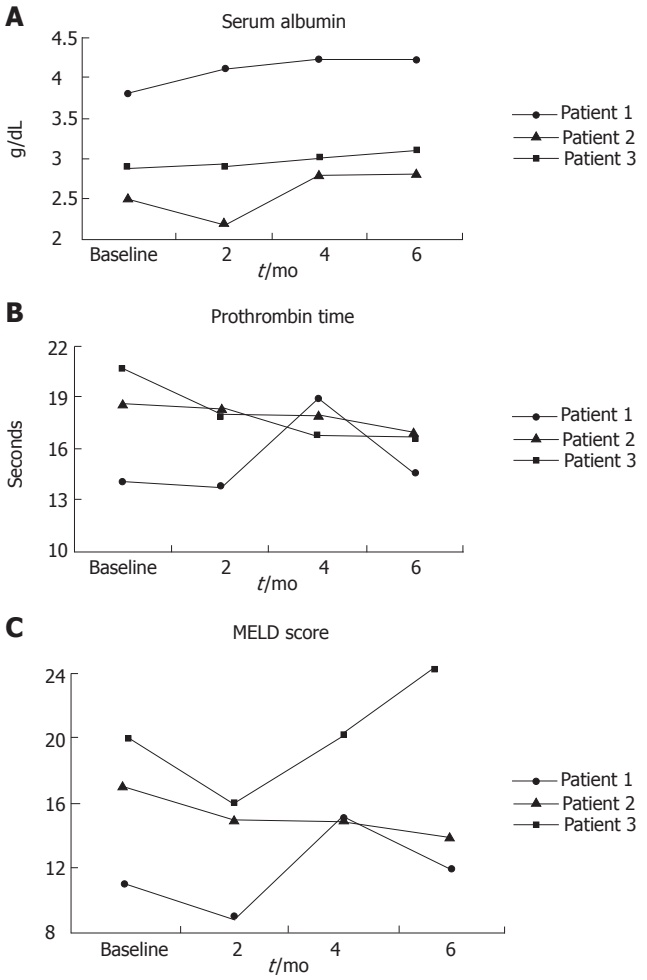

Figure 1 Changes in serum albumin (**A**), prothrombin time (**B**), and MELD score (**C**) during the study period.

urgent liver transplantation. After that, we prematurely stopped the project. That patient had tense ascites and serum creatinine of 1.37 g/dL before undergoing the procedure. She probably had some degree of type 2 hepatorenal syndrome at baseline, and radiocontrast nephropathy was a triggering factor to progress to type 1 hepatorenal syndrome. We used nonionic low osmolal radiocontrast agents which reduce the risk of contrast nephropathy in those who are at increased risk for this condition[13,14]; although such adverse effects could not be prevented in our patient. We suggest that other routes of cell infusion (e.g. other than hepatic artery) be considered in the future studies. An alternative method would be to use carbon dioxide to visualize hepatic artery[15].

Recently, Gasbarini *et al*[16] reported successful portal infusion of $CD34^+$ stem cells in a case of drug-induced acute liver failure. Also, Gordon *et al*[12] injected autologous $CD34^+$ stem cells through hepatic artery or portal vein in 5 patients with cirrhosis. In that study, the procedure was safe, and 3 of 5 patients showed improvement in serum bilirubin, and 4 of 5 in serum albumin after two months follow up. Accordingly, in our study, we observed some improvements in the serum albumin and PT in 2 of 3 patients at 6 mo of follow up. However, we are not sure if such improvement was related to HSCs infusion or simply related to the natural course of the disease in the patients.

Although transplanted HSCs can generate hepatocytes, it seems that it is a rare event[17]. There are controversies in the mechanisms by which HSCs generate hepatoytes. While, Wang *et al*[18] reported that cell fusion is the principle source of bone-marrow-derived hepatocytes; Jang, *et al*[19] reported HSCs convert into liver cells within days without fusion. The exact therapeutic role of HSCs in liver cirrhosis should be evaluated in further controlled trials.

In our study, there was significant improvement in the quality of life of all three patients at the end of follow up. Such improvement may be related to the improvement of liver function, or may be due to a placebo effect.

We have performed another phase 1 study of mesenchymal stem cell (MSC) transplantation in cirrhosis (Mohamadnejad *et al* 2006; Submitted for publication). The results of our MSC transplantation were more promising than this study of hematopoietic stem cell transplantation. However, the efficacy of each type of bone marrow stem cells in the improvement of liver function and quality of life of the patients should be evaluated in further controlled trials.

One of the limitations of our work was the fact that we did not track the infused HSCs in the patients' bodies. Although tracing of infused stem cell in the body seems to be a complicated issue and the interpretation of tracing studies has recently created a lot of controversies[20,21], it is very important to understand the way stem cells act to improve liver function. We are now planning to trace the stem cells in our future studies.

In conclusion, we found that infusion of HSCs through the hepatic artery in decompensated cirrhosis may marginally improve liver functions in some patients; however, it may cause major side effects in the patients. In fusion of HSCs through the hepatic artery may best be avoided in further trials. Although, this study does not preclude infusion of $CD34^+$ stem cells through other routes.

ACKNOWLEDGMENTS

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RAPID COMMUNICATION

The receptor for β2GP Ⅰ on membrane of hepatocellular carcinoma cell line SMMC-7721 is annexin Ⅱ

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Abstract

AIM: To evaluate the receptor protein which can specifically bind to β2GP Ⅰ on the membrane of hepatocellular carcinoma (HCC) cell line SMMC-7721, and to study the biological function of the receptor.

METHODS: Through β2GP Ⅰ-affinity chromatography column, the peptid-polysome-mRNA complex, which can specially bind to β2GP Ⅰ, stayed with the column and was separated from the whole polysome of liver cells, and then eluted and collected. Using cDNA synthesis kit and cDNA PCR kit, the corresponding cDNA was obtained and sequenced. RT-PCR was used to amplify annexin Ⅱ, and flow cytometry was used to study the competitive binding of annexin Ⅱ with β2GP Ⅰ to SMMC-7721.

RESULTS: A total of 1.1 kb of the cDNA fragment of the specific binding protein of β2GP Ⅰ on liver cell membrane was obtained. The sequence of cDNA shared high homology with human annexin Ⅱ (98%). Annexin Ⅱ was expressed on the membrane of SMMC-7721, and could compete with β2GP Ⅰ for combining with SMMC-7721.

CONCLUSION: The receptor for β2GP Ⅰ on membrane of SMMC-7721 cells is annexin Ⅱ, which might bridge HBV to infect hepatocytes.

Key words: β2-Glycoprotein Ⅰ; Hepatocellular carcinoma cell, Human annexin Ⅱ

Gao PJ, Shi Y, Gao YH, Liu YW, Tan Y. The receptor for β2GP Ⅰ on membrane of hepatocellular carcinoma cell line SMMC-7721 is annexin Ⅱ. *World J Gastroenterol* 2007; 13(24): 3364-3368

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INTRODUCTION

β2-Glycoprotein Ⅰ (β2GP Ⅰ) is an abundant plasma glycoprotein. Because of its high affinity binding with plasma phosphorlipid, it is also named apolipoprotein H. It has been shown that β2GP Ⅰ could act as anticoagulant[1] and is an important autoantigen in the antiphospholipid antibody syndrome[2,3]. Up to now, it has been found that β2GP Ⅰ has many other functions. In 1994, Mehdi *et al*[4] demonstrated that β2GP Ⅰ was capable of binding to recombinant hepatitis B surface antigen (rHBsAg), suggesting that β2GP Ⅰ may facilitate entry of the virus into hepatocytes. They also found that rHBsAg bound to β2GP Ⅰ very poorly if β2GP Ⅰ was coated directly on a microtiter well, or if it was presented in a soluble form. While binding was 100-fold more efficient when β2GP Ⅰ was presented as a complex with monoclonal antibody (mAb) P2D4. These results suggest that chemical modification of β2GP Ⅰ makes it highly reactive with rHBsAg[5]. Recently it has been reported that β2GP Ⅰ-HBsAg combining was relative to the presence of hepatitis B virus markers and β2GP Ⅰ binding activity for HBsAg was higher in sera from patients in the active virus replication phase[6].

We have previously finished purification and evaluation of formation of β2GP Ⅰ, and found that the level of anti-β2GP Ⅰ antibodies in patients with chronic hepatitis B and post-hepatitis B cirrhosis was significantly increased, thereby suggesting that β2GP Ⅰ can take part in HBV infection. In our previous studies, we also verified that β2GP Ⅰ could specifically bind to rHBsAg[7,8]. Through ligand blot analysis, fluorescence microscope and flow cytometry, it was probably the first time to prove that there exists a protein on SMMC-7721 cell membrane that can bind to β2GP Ⅰ with specificity[9]. We concluded that the protein may be the receptor of β2GP Ⅰ, and it might be a carrier which can bridge HBV to invade hepatocytes. In this study, we will evaluate the receptor on SMMC-7721 cell line that can bind to β2GP Ⅰ with specificity.

MATERIALS AND METHODS

Preparation of β2GP Ⅰ-affinity chromatography column

Purified β2GP Ⅰ was preserved in our laboratory.

According to instructions of Epoxy-activated Sepharose 6B, the medium was suspended in distilled water. The purified β_2GP I was dissolved in buffer solution, mixed with gel granules for 16 h with shake cultivation at 37℃, and then was packed in column. The column was washed with buffer solution, distilled water, buffer solution A and buffer solution B by turns in order to eliminate excess β_2GP I . The remained active radical was blocked with 1 mol/L ethanolamine at 37℃. Protein in the collected elutriant was quantitated with BCA methods.

Extraction of polysome of liver cells

All procedures were performed at 4℃. Under aseptic conditions, connective tissue and fat was eliminated from liver with scissors. Then the liver tissue was cut into scraps and grinded in cell homogenizer. After being filtered through stainless steel screen, the products were washed with solution A (pH 8.0, 60 mmol/L sodium phosphate buffer solution, 45 mmol/L NaCl, 55 mmol/L glycose, 1 μg/mL cycloheximide). The liver cells were counted, and 2×10^{10} cells were resuspended in solution B (pH 8.0, 60 mmol/L sodium phosphate buffer solution, 45 mmol/L NaCl, 55 mmol/L glycose, 1 μg/mL cycloheximide, 40 U/mL heparin, 10 mmol/L DTT). Then 10 mL of solution C (pH 7.8, 50 mmol/L Tris-HCl, 300 mmol/L NaCl, 10 mmol/L $MgCl_2$, 1 μg/mL cycloheximide, 20 U/mL heparin) and nonidet P-40 were added slowly to a final concentration of 3.5 mL/L in order to split them. After incubation for 5 min and centrifugation, the supernatant was collected. Sodium deoxycholate of final concentration of 3.5 mL/L was added to destroy microsome completely. Remainder of supernatant was loaded at 65% (W/W) sucrose in Buffer D (pH 7.6, 25 mmol/L Tris-HCl, 150 mmol/L NaCl, 5 mmol/L $MgCl_2$, 20 U/mL heparin), then ultracentrifuged at 5000 r/min for 2 h. The deposition was resuspended in solution E (pH 7.6, 25 mmol/L Tris-HCl, 150 mmol/L NaCl, 5 mmol/L $MgCl_2$, 1 mL/L nonidet P-40, 1 μg/mL cycloheximide, 20 U/mL heparin), and stored in liquid N_2.

Affinity purification of mRNA of the receptor protein

According to manufacturer's instructions, the β_2GP I -affinity chromatography column was balanced with solution A. The polysome extraction was slowly loaded on the column repeatedly. After being processed for 1 h, the column was washed with 20-30 volume solution E, eluted with solution F (pH 7.6, 25 mmol/L Tris-HCl, 150 mmol/L EDTA, 20 U/mL heparin) in order to collect the objective mRNA which can specifically bind β_2GP I , and then 0.5 mol/L NaCl and 1 g/L SDS were added. The above solution was loaded on oligo-dT cellulose column. The oligo-dT cellulose column was washed with solution G (pH 7.6, 25 mmol/L Tris-HCl, 500 mmol/L NaCl, 1 g/L SDS, 20 U/mL heparin), then eluted with solution G without NaCl. The elutriant containing objective mRNA was concentrated, washed, dissolved in sterile water, and stored at -80℃.

Synthesis of double strands cDNA

Synthesis of 1st strand cDNA: mRNA was treated at 65℃ for 5 min, and ice bathed immediately. A total volume of 10 μL of reaction mixture contained 2 μL of sample mRNA, 2 μL of 5 × 1st Strand Synthesis buffer, 1 μL of dNTP, 1 μL of RNase inhibitor, 1 μL of oligo dT-RA primer, 1 μL of RAV-2 reverse transcriptase, 2 μL of DEPC H_2O. The reverse transcription was performed for 10 min at 30℃, for 1 h at 42℃, and finally for 5 min at 80℃.

Synthesis and external smoothing of 2nd strand cDNA: In aforementioned reaction mixture, 10 μL of 5 × 2nd Strand Synthesis buffer, 20.5 μL of DEPC H_2O and 1.0 μL of *E. coli* DNA Ligase Mixture were added and mixed gently. After incubation for 1 h at 12℃, 1 h at 22℃, and 10 min at 70℃ in turn, the mixture was mixed gently with 2 μL of T4 DNA PolyMerase I and kept at 37℃ for 10 min. Then 4 μL of Stop Solution was added to stop the reaction.

Purification of double strand cDNA: The obtained cDNA was extracted with phenylic alcohol/chloroform, precipitated with isopropanol, washed with ethanol and dried.

Ligation of cassete adaptor

After being dissolved in 5 μL of sterilized water, the above sediment was mixed with 2 μL CA cassette adaptor and 6 μL of ligation solution gently. The reaction remained at 16℃ for 30 min. Then 25 μL of 4 mol/L ammonium acetate was added. The products was precipitated with isopropanol and then dissolved in 30 μL of sterilized water at -80℃.

PCR of the double strand cDNA

A total volume of 50 μL of PCR mixture contained 30 μL of prepared cDNA solution, 5 μL of 10 × Ex Taq buffer, 4 μL of dNTP mixture, 0.5 μL of RA primer, 0.5 μL of CA primer, 0.25 μL of Takara Ex Taq and 9.75 μL of sterilized water. PCR mixture was subjected to pre-denaturation at 94℃ for 1 min, followed by 35 amplification cycles of denaturation at 94℃ for 30 s, primer annealing at 60℃ for 30 s and extension at 72℃ for 3 min. Finally, the PCR mixture incubated at 72℃ for 5 min, reaction was held at 4℃.

Sequencing

According to molecular clone, the PCR products were purified and then linked to TA vector. The recombined plasmid was transfected into receptive *E. coli* DH5α. Through blue-white screening, the positive colony was obtained. Using plasmid extraction kit, the recombined plasmid was extracted, and sequenced in the company. Finally, the sequence was analyzed using BLAST.

Amplification of annexin Ⅱ from liver tissue by RT-PCR

According to manufacturer's instruction (Invitrogen Company), using Trizol reagent, total RNA was isolated from cultured 1×10^7 SMMC-7721 cells and stored at -70℃. In accordance with the instructions of AMV reverse transcriptase, 20 μL of mixture, containing 20 μg of total RNA, 2 μL of oligo (dT) and 18 μL of DEPC H_2O, was kept in a 200-μL micro-centrifuge tube, mixed

gently and then heated at 70℃ for 5 min. After immediate cooling on ice for at least 5 min, 5 μL of dNTP mixture, 10 μL of AMV 5 × buffer, 2 μL of Rnasin, 3 μL of AMV reverse transcriptase and 10 μL of DEPC H_2O were added to reach a total volume of 50 μL, followed by incubation at 42℃ for 90 min, and then for 5 min at 95℃ for inactivation. Thus the RNA was reverse transcribed into cDNA which was stored at -70℃. Referring to mRNA of annexin Ⅱ from GenBank and following principle of design for primers, a pair of primers was designed: AAAAGATCTCCAGCTTCCTTCAAA (sense); AAAGTCGACATTTCTGGACGCTCA (anti-sense). The reaction system, containing 5 μL of 10 × buffer, 5 μL of Mg^{2+}, 4 μL of dNTP, 1 μL of each sense and antisense primers, 10 μL of cDNA, 1 μL of Taq enzyme and 23 μL of DEPC H_2O, was subjected for 40 amplification cycles of denaturation at 94℃ for 1 min, annealing at 55℃ for 1 min and extension at 72℃ for 2 min, followed by a final extension at 72℃ for 7 min. Ten microliters of PCR product was electrophoresed on 10 g/L agarose gel.

Flow cytometry

Green fluorescent protein (GFP) and β2GP I labeled with GFP (GFP-β2GP I) were gifted by Central Laboratory of our hospital. SMMC-7721 cells are preserved in our laboratory. About 9×10^6 of SMMC-7721 cells were collected, washed and divided averagely into 9 tubes. These 9 tubes were randomly divided into three groups, 3 tubes in each group: group A, group B and group C. After being washed twice with PBS, each tube of cells was added with 2 mL of BD FACS Permeabilizing Solution and kept at room temperature for 10 min. Then they were centrifuged to discard the supernatant. The cells were washed with PBS again, and added with 0.45 mL of buffer solution containing Ca^{2+}. Thereafter, groups A, B and C were added with 0 μL, 0 μL and 10 μL of annexin Ⅱ, respectively and kept at room temperature for 30 min. Finally, 0.05 mL of GFP was added to the each tube of group A, while 0.05 mL of GFP-β2GP I to each tube of groups B and C. All tubes were kept at room temperature for 2 h and washed twice with PBS, and then cells of each tube were suspended in 0.4 mL of PBS and detected using flow cytometry.

RESULTS

Couple rate of prepared β2GP Ⅰ -affinity chromatography column

Ten milligrams of purified β2GP I was dissolved in couple solution and mixed with gel granule for 16 h with shake cultivation at 37℃. The excess β2GP I was washed with couple solution, distilled water, buffer solution A and buffer solution B by turns and then was collected. The quantity of excess protein in the eluted solution was measured, which was 1.1 mg. So the couple rate was 89%. The couple efficiency was good enough to be used for the latter study.

Electrophoresis of RT-PCR products

With the β2GP I -affinity chromatography column prepared by ourselves, the peptid-polysome-mRNA which can specially bind to β2GP I conjugated with β2GP I on the column, and then was eluted. Through cDNA synthesis kit, the first strand cDNA was obtained. Then the double strand cDNA was acquired. Agar gel electrophoresis confirmed that the molecular weight of the cDNA fragment was 1.1 kb (Figure 1).

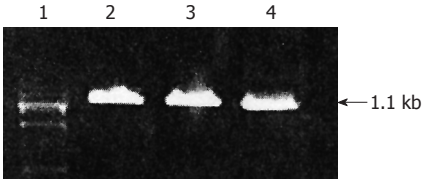

Figure 1 Agarose gel electrophoresis of RT-PCR products. Lane 1: DNA maker; Lanes 2, 3, 4: PCR products.

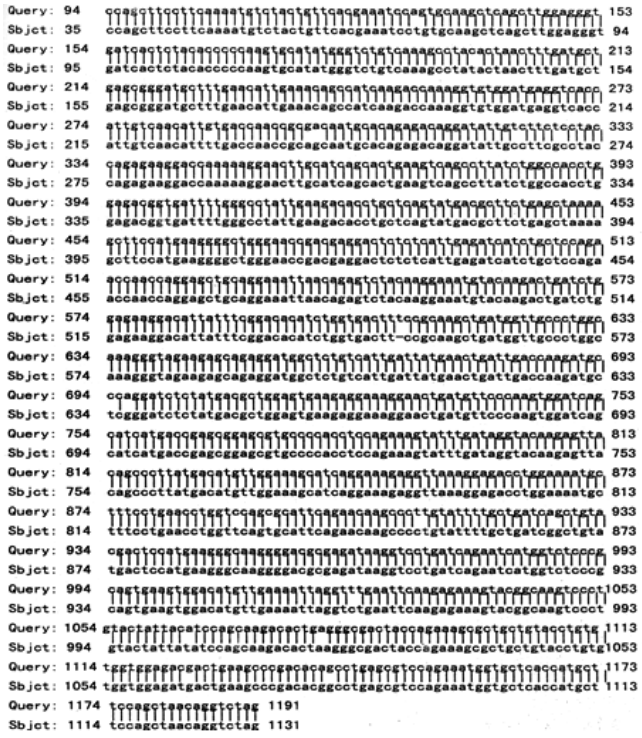

Figure 2 Comparison of the cDNA sequence of receptor of β2GP Ⅰ with annexin Ⅱ.

Gene sequencing and analysis of β2GP Ⅰ -binding receptor

The recombined plasmid purified from positive cloned bacteria was sequenced in Beijing Dingguo Biological Technique Company. Then the sequence was analyzed for its homology with GenBank BLAST. The results showed that the sequence of cDNA shared high homology with human annexin Ⅱ (98%) (Figure 2).

RT-PCR of annexin Ⅱ

RT-PCR was used to detect the expressions of β-actin and annexin Ⅱ in HUVEC, SMMC-7721 and hepatoma tissue (Figure 3). Lanes 3, 5 and 7 show β-actin expression. It showed that the total mRNA of the three groups had been extracted successfully. Lanes 2, 4 and 6 show a single band of 1000 bp which represents annexin Ⅱ. Similarly, HUVEC, SMMC-7721 and hepatoma tissue expressed annexin Ⅱ.

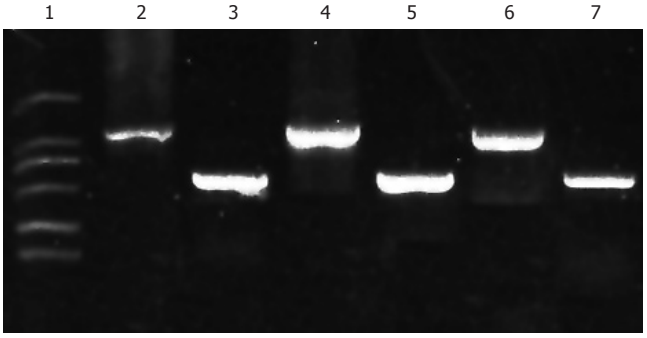

Figure 3 Electrophoregram of RT-PR product of annexin Ⅱ. Lane 1: DNA marker DL 2000 (from top to bottom: 2000, 1000, 750, 500, 250, 100 bp); Lanes 2, 4 and 6: Annexin Ⅱ from HUVEC, SMMC-7721 and HCC sample, respectively; Lanes 3, 5 and 7: β-actin RT-PCR product from HUVEC, SMMC-7721 and HCC sample, respectively.

Competitive inhibition

Using flow cytometry, we proved that β2GP Ⅰ labeled with GFP could bind to SMMC-7721. The binding rate of β2GP Ⅰ-GFP with SMMC-7721 (66.81%) was significantly higher than that of GFP with SMMC-7721 (1.16%) (Figure 4). Once β2GP Ⅰ-GFP had been incubated with annexin Ⅱ at room temperature in advance, the binding rate of β2GP Ⅰ-GFP with SMMC-7721 dropped to 7.21% (Figure 4C). Thus these results suggested that annexin Ⅱ could inhibit the combination of β2GP Ⅰ with SMMC-7721.

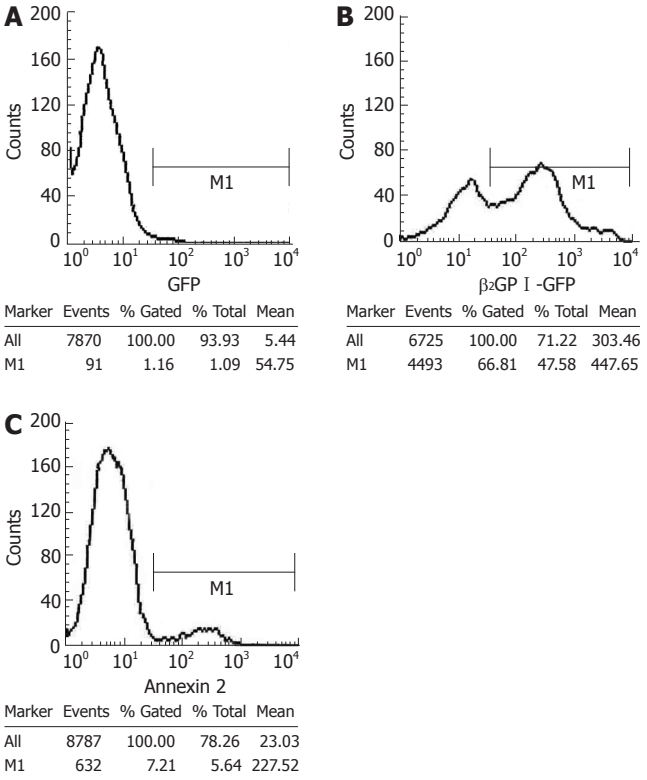

Figure 4 FACS analysis revealed descent of the binding rate of GFP-β2GP Ⅰ with SMMC-7721 after pretreatment with annexin Ⅱ.

DISCUSSION

Through cloning, recombining and sequencing the gene of β2GP Ⅰ-bound receptor on membrane of SMMC-7721 cells, we found that the gene fragment of the receptor shared high homology with human annexin Ⅱ (98%). Moreover, annexin Ⅱ was found to exist on the membrane of SMMC-7721 by RT-PCR. At the same time, we validated that annexin Ⅱ could compete with β2GP Ⅰ for combining SMMC-7721. Thus, it can be concluded that the receptor specific for β2GP Ⅰ might be annexin Ⅱ.

Annexin Ⅱ (*M*r-36 ku) belongs to a family of Ca^{2+}-dependent membrane-binding proteins encoded by some 20 different genes[10-12]. Annexins are structurally related proteins, each of which consists of an *N*-terminal "tail" and *C*-terminal "core" domain. The core domains of different annexins are highly conserved and share 40%-70% homology. Usually annexin Ⅱ binds to S100A10 (p11), a Ca^{2+}-modulated protein, to form tetramer. P11 can adulate the binding activity of annexin Ⅱ for calcium ion or phospholipids. It has been shown that the gene of annexin Ⅱ lies on the 15th chromosome, and has a 1.4-kb conserved encoding sequence. Despite the lack of a hydrophobic signal peptide, the presence of annexin Ⅱ on cell surfaces is well established. It has been proven that annexin Ⅱ is affluent on endotheliocyte, monocyte/macrophage, myeloid cell and some tumor cells. And approximately 4.3% of total endothelial annexin Ⅱ is associated with the phospholipids on the external plasma membrane. Annexin II has been implicated to possess many biological functions in a variety of physiologic processes, such as anti-inflammatory effect of glucocorticoid, calcium-dependent exocytosis, immune response, calcium transport and phosphorlipase A2 regulation[12]. Recently, research has suggested that annexin Ⅱ is an endothelial cell receptor for tissue-type plasminogen activator (t-PA) and plasminogen (PLG)[13-18] and can activate them. So under normal conditions, annexin Ⅱ is an important modulation receptor in coagulation-anticoagulation-fibrinogenolysis system. Over-expression of annexin Ⅱ will evoke hyperfunction of PLG and cause thrombosis and hemorrhage. As annexin Ⅱ can act as second messenger in the modulation path of cell division, it has also been found to be related to cell proliferation and tumor growth. In addition, it has been suggested that the expression disturbance of annexin Ⅱ in many kinds of cancers accelerate carcinogensis and metastasis.

With respect to the relationship between annexin Ⅱ and virus infection, it has been elucidated that annexin Ⅱ can serve as a receptor for cytomegalovirus and mediate its infection[19]. Until now, there lacks report on the relationship between annexin Ⅱ and HBV infection. Our preliminary studies suggest that annexin Ⅱ is the receptor of β2GP Ⅰ, and β2GP Ⅰ can bind to HBsAg. Therefore, we speculate that annexin Ⅱ might have a potential role in HBV infection. To evaluate validity of the hypothesis and make sure which domain of annexin Ⅱ binds to β2GP Ⅰ so as to bridge HBV infection, we will use phage display in our further study.

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RAPID COMMUNICATION

Difference in gene expression of macrophage between normal spleen and portal hypertensive spleen idendified by cDNA microarray

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Abstract

AIM: To identify the difference in gene expression of microphage (Mφ) between normal spleen and portal hypertensive spleen using cDNA microarrays and find new gene functions associated with hypersplenism in portal hypertension.

METHODS: The Biostar-H140s chip containing 14112 spots of cDNAs were used to investigate the difference of the expression. The total RNA extracted from macrophages isolated from both normal spleen and portal hypertensive spleen was reversely transcribed to cDNA with the incorporation of fluorescent (cy3 and cy5) labeled dCTP to prepare the hybridization probes. After hybridization, the gene chip was scanned for the fluorescent intensity. The differentially expressed genes were screened. That was repeated three times, and only the genes which had differential expression in all three chips were considered to be associated with hypersplenism in portal hypertension.

RESULTS: Eight hundred and ninety-six, 1330 and 898 genes were identified to be differentially expressed in three chips, respectively. One hundred and twenty-one genes (0.86%) were identified to be differentially expressed in all three chips, including 21 up-regulated genes and 73 down-regulated genes. The differentially expressed genes were related to ionic channel and transport protein, cyclin, cytoskeleton, cell receptor, cell signal conduct, metabolism, immune, and so on. These genes might be related to the hypersplenism in portal hypertension.

CONCLUSION: The investigations based on cDNA microarray can screen differentially expressed genes of macrophages between normal spleen and portal hypertensive spleen, thus may provide a new idea in studying the pathogenesis of hypersplenism in portal hypertension.

Key words: Hypersplenism; Macrophage; cDNA microarray

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INTRODUCTION

It is reported that, compared with the macrophage (Mφ) in normal spleen, the Mφ in portal hypertensive spleen has a large amount of acid phosphatase, lysosome and pseudopodium, and can destruct much more erythrocytes and thrombocytes. This proved that the destruction of hemocytes by Mφ of spleen plays an important role in the development of hypersplenism in portal hypertension[1,2]. Our previous studies suggested that phagocytosis of Mφ was augmented in hypersplenism in portal hypertension; however, the specific mechanisms are not clear. In this study, cDNA microarrays were used to detect the difference in gene expression of Mφ between normal spleen and portal hypertensive spleen and find new gene functions associated with hypersplenism in portal hypertension in an attempt to explore the pathogenesis of hypersplenism in portal hypertension.

MATERIALS AND METHODS

Materials

The excised human spleen specimens used in this study were provided with the approval of the hospital authorities. The experimental group included 3 cases of excised human spleen of portal hypertension and hypersplenism (all 3 cases had chronic hepatitis B), and the

control group included 2 cases of excised human spleen of traumatic splenic rupture.

Mφ isolation and purification and total RNA extraction

Mφ was isolated and purified by adherent culture[3]. Total RNA was extracted from Mφ by the TRIzol method[4].

Construction of cDNA microarray

The Biostar-H140s cDNA microarray provided by Shanghai BioStar Genechip Inc., consists of a total of 14112 human genes. The cDNA inserts were amplified using the polymerase chain reaction (PCR) with universal primers, and then purified according to standard method. All PCR products were examined by agarose gel electrophoresis to ensure the quality. Then the amplified PCR products were dissolved in a buffer solution. The solution with amplified PCR products were spotted onto silylated slides (TeleChem International, USA) using a Cartesian PixSys 7500 motion control robot (Cartesian Technologies, USA). Glass slides with spotted cDNA were hydrated for 2 h in 700 mL/L humidity, dried for 0.5 h at room temperature, and UV crosslinked (65 mj/cm). They were further processed at room temperature by soaking in 2 g/L sodium dodecyl sulfate (SDS) for 10 min, in distilled H_2O for 10 min, and 2 g/L sodium borohydride (NaBH4) for 10 min. The slides were dried again and ready for use.

Probe preparation

The fluorescent cDNA probes were prepared through reverse transcription and then purified according to the protocol of Schena[5]. The total RNA of Mφ was extracted from 2 cases of normal spleen respectively, and then was mixed as the control group. The total RNA of Mφ was extracted from 3 cases of portal hypertensive spleen respectively, and each case was treated as the experimental group. The probes from the total RNA of control group was labeled with Cy3-dUTP, while those from the total RNA of experimental group were labeled with Cy5-dUTP. The probes were then mixed, precipitated and resolved in a hybridization buffer.

Hybridization and washing

Microarrays were pre-hybridized with hybridization solution containing 0.5 g/L denatured salmon sperm DNA at 42℃ for 6 h. Fluorescent probe mixtures were denatured at 95℃ for 5 min, and the denatured probe mixtures were applied onto the pre-hybridized chip under a cover glass. Chips were hybridized at 42℃ for 16-18 h. The hybridized chips were then washed at 60℃ for 10 min each in the mixture of 5 mL/L solution 1 and 20 mL/L solution 2, and 50 mL/L solution 3, then dried at room temperature for scanning (all reagents used in this procedure were contained in the Chip Hybridization Kit provided by Shanghai BioStar Genechip Inc.).

Detection and analysis

The chips were scanned with a ScanArray 4000 (Packard Biochip Technologies, USA) at two wavelengths to detect emission from both Cy3 and Cy5. The acquired images were analyzed using QuantArray software (Packard Biochip Technologies, USA). Ratios of Cy5 to Cy3 were computed for each location on each microarray. Overall intensities were normalized with a correction coefficient obtained using the ratios of 96 housekeeping genes in each chip. The intensities of each spot at the two wavelengths represent the quantity of Cy3-dUTP and Cy5-dUTP, respectively, hybridized to each spot. Thus, the ratio of each spot represents the ratio of mRNA expression abundance between the gene of Mφ in normal spleen and portal hypertensive spleen. The detection results were described in both scanned microarray images and microarray scatter plots. That was repeated three times, and only the genes that had differential expression in all three chips were considered associated with hypersplenism in portal hypertension.

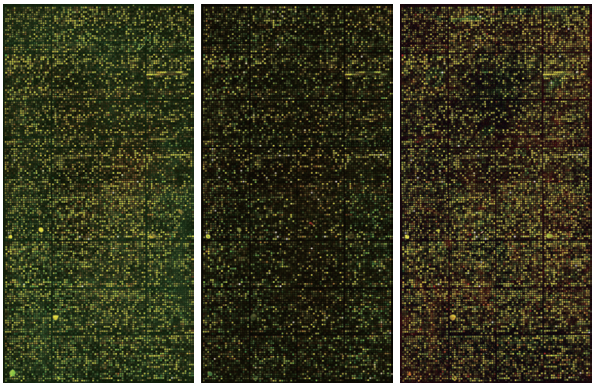

Figure 1 Scanning results of hybridized signals on gene chip. Color of spots in image: high expression (red), low expression (green) and no change in expression (yellow).

RESULTS

Scanned microarray images

In the scanned microarray images (Figure 1), red points represent the higher expression genes of Mφ in portal hypertensive spleen than those in normal spleen, green points represent the lower expression genes, and yellow points represent the genes that have no change in expression. The hybridization signal of chips is distinct and balanced, indicating that the results are reliable. Compared with the genes of Mφ in normal spleen, a few genes of Mφ in portal hypertensive spleen were highly expressed, some were lowly expressed, however most genes showed no change in expression.

Microarray scatter plots

As indicated in the microarray scatter plots (Figure 2), most genes show a concentrated pattern surrounding the diagonal (red points), which means that the ratios range from 0.5 to 2.0, and there is no difference in the gene expression between normal spleen and portal hypertensive spleen. However, the other genes are away from the diagonal (yellow points), indicating that the ratios are beyond the range of 0.5-2.0, and the difference in the expression of those genes is not significant between normal spleen and portal hypertensive spleen.

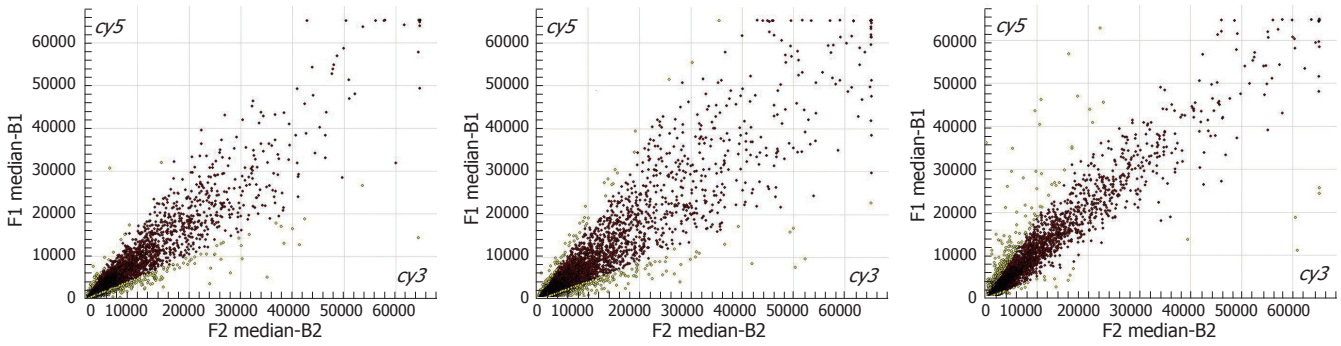

Figure 2 Scatter plots of hybridized signals on gene chip.

Table 1 Differentially expressed genes up-regulated in macrophages of portal hypertensive spleen

GenBank No.	Gene name and description	Average ratio
AF189009	UBQLN2, ubiquilin 2	2.117
BX537509	NET1, neuroepithelial cell transforming gene 1	2.270
NM_004830	CRSP3, cofactor required for Sp1 transcriptional activation	2.334
AF025771	ZNF189, zinc finger protein 189	2.453
AK090727	Homo sapiens cDNA FLJ33408 fis, clone BRACE2010550	2.511
BX537955	TRIM37, tripartite motif-containing 37	2.553
NM_006716	ASK, activator of S phase kinase	2.620
NM_181523	PIK3R1, phosphoinositide-3-kinase, regulatory subunit 1	2.755
XM_376537	Homo sapiens BCL2-associated transcription factor 1 (BCLAF1), mRNA	2.993
NM_004416	DTX1, deltex homolog 1 (Drosophila)	3.553

Table 2 Differentially expressed genes down-regulated in macrophages of portal hypertensive spleen

GenBank No.	Gene name and description	Average ratio
BC068441	IL1RN, interleukin 1 receptor antagonist	0.179
NM_014909	KIAA1036	0.268
BU732296	Homo sapiens cDNA clone UI-E-CI1-afo-e-04-0-UI 3', mRNA sequence	0.275
AK092248	Homo sapiens cDNA FLJ34929 fis, clone NT2RP7004728	0.293
NM_006254	PRKCD, protein kinase C, delta	0.298
BX648172	OAZ2, ornithine decarboxylase antizyme 2	0.302
BM542499	Homo sapiens cDNA clone IMAGE:5521023 5', mRNA sequence	0.311
BF240734	Homo sapiens cDNA clone IMAGE:4091885 5', mRNA sequence	0.324
NM_003902	FUBP1, far upstream element (FUSE) binding protein 1	0.338
BM993772	Homo sapiens cDNA clone IMAGE:5869020 3', mRNA sequence	0.344
NM_005373	MPL, myeloproliferative leukemia virus oncogene	0.359
BX647757	SCML1, sex comb on midleg-like 1 (Drosophila)	0.370
NM_005781	ACK1, activated Cdc42-associated kinase 1	0.381
AL832249	Homo sapiens mRNA; cDNA DKFZp686P1077	0.390
AK092130	LOC285378, hypothetical protein LOC285378	0.406
AF466367	Homo sapiens clone KU011197 unknown mRNA	0.434
NM_001067	TOP2A, topoisomerase (DNA) Ⅱ alpha	0.437
AF051151	TLR5, toll-like receptor 5	0.452

Differentially expressed genes

There were 896, 1330 and 898 genes identified to be differentially expressed in three chips, respectively; 121 genes (0.86%) were differentially expressed in all three chips, including 95 genes which could be found in the GenBank, the other 26 genes were not reported and probably were unidentified novel genes. Among 95 known genes, 1 gene (GenBank No: NM_012218) was related to hepatitis B, and the other 94 genes might be those that were differentially expressed between the Mφ in normal spleen and the Mφ in portal hypertensive spleen, including 21 up-regulated known genes and 73 down-regulated known genes. Ten differentially expressed genes that were up-regulated in macrophges of portal hypertensive spleen are listed in Table 1 and 18 differentially expressed genes that were down-regulated are demonstrated in Table 2.

DISCUSSION

Since the microarray analysis was first reported by Schena[6] in 1995, gene chips have been widely used in studying the functions of genes. The results of this study proved that gene chips can successfully profile changes in gene expression on a genomic scale with low consuming, high sensitivity and high-flux. In this study, cDNA microarrays were used to detect the difference in gene expression of Mφ between normal spleen and portal hypertensive spleen and find new gene functions associated with hypersplenism in portal hypertension in an attempt to explore the pathogenesis of hypersplenism in portal hypertension. No similar study has been reported until now.

In order to obtain enough amounts of total RNA and eliminate individual variation, the total RNA of Mφ extracted from 2 cases of normal spleen respectively was mixed as the control group, and then was matched with that of 3 cases of the experimental group to 3 match-pairs for the cDNA microarray analysis. The differentially expressed genes were found to be related to ionic channel and transport protein, cyclin, cytoskeleton, cell receptor, cell signal conduct, metabolism, immune, and so on.

PRKCD encoding protein kinase C delta[7] was found down-regulated in the Mφ of portal hypertensive spleen. Protein kinase C delta plays an important role in regulating IL-13-induced 15-lipoxygenase (15-LO) expression in human monocytes and subsequently modulates the inflammatory responses mediated by 15-LO products[8]. Besides, protein kinase C delta is related to monocytic differentiation[9,10]. Those findings indicate that PRKCD has a close relationship with the function of human monocytes (including Mφ). The mature dendritic cell (DC) is considered to be the most potent antigen-presenting cell. Regulation of the DC, particularly its survival, is therefore critical. Bertho *et al*[11] found that MHC class Ⅱ-mediated apoptosis of mature DC is produced by activation of the protein kinase C delta isoenzyme. Thrombin can stimulate the production of vascular adhesion molecule-1 (VCAM-1) in endothelial cells, however, it is found to be mediated by the signaling pathways invoved with protein kinase C delta[12]. These findings indicate that PRKCD plays an important role in inducing apoptosis and producing cytokines. However, the effects of down-regulated PRKCD on the Mφ of portal hypertensive spleen remain to be further investigated. IL-1 is an important mediator of inflammation and tissue damage in multiple organs in both experimental animal models and humans[13-15]. The balance between IL-1 and IL-1Ra (interleukin 1 receptor antagonist, IL-1Ra) in local tissues plays an important role in the susceptibility to and severity of many diseases[16,17]. Treatment of rheumatoid arthritis (RA) with daily subcutaneous injections of recombinant IL-1Ra protein has been shown to be efficacious. Gene therapy with IL-1Ra is being evaluated for the treatment of RA and other human diseases[18]. IL1RN encoding IL-1Ra was found down-regulated significantly (the average ratio was 0.179) in the Mφ of portal hypertensive spleen. This leads to the imbalance between IL-1 and IL-1Ra, and it might be related to the pathogenesis of hypersplenism in portal hypertension, but the specific mechanisms need to be further studied.

ASK encoding activator of S phase kinase was found up-regulated in the Mφ of portal hypertensive spleen. Cdc7-Dbf4 kinase complexes, conserved widely in eukaryotes, play essential roles in initiation and progression of the S phase. Cdc7 kinase activity fluctuates during cell cycle, and this is mainly the result of oscillation of expression of the Dbf4 subunit. Yamada *et al*[19] had isolated and characterized the promoter region of the human ASK gene encoding Dbf4-related regulatory subunit for human Cdc7 kinase, and identified one ASK promoter segment, which was sufficient for mediating growth stimulation. In the Mφ of portal hypertensive spleen, the up-regulation of ASK may lead to the activity enhancement (including phagocytosis) of Mφ, resulting in the pathogenesis of hypersplenism in portal hypertension. Phosphatidylinositol 3-kinase (PIK3) is a key step in the metabolic actions of insulin. One 85 KDa regulatory subunit of PIK3 is encoded by PIK3R1 (phosphoinositide-3-kinase, regulatory subunit 1). It was proved that the expression of PIK3R1 was associated with alterations in glucose/insulin homeostasis[20]. In our study, PIK3R1 was found up-regulated significantly in the Mφ of portal hypertensive spleen, indicating that more insulin existed and the glycometabolism was enhanced in Mφ. Furthermore, enhancement of glycometabolism is regarded as an index of enhanced cell functions, therefore we presume that the up-regulation of PIK3R1 may cause the functional enhancement of Mφ in spleen. However, the possible molecular mechanisms remain undiscovered.

Many differentially expressed genes of Mφ between normal spleen and portal hypertensive spleen have been successfully screened by cDNA microarrays, providing clues and target genes in studying the molecular mechanisms of pathogenesis of hypersplenism in portal hypertension. However, the implication of the gene expression needs to be further investigated.

COMMENTS

Background

The destruction of hemocytes by macrophage of spleen plays an important role in the development of hypersplenism in portal hypertension. The authors have proved that phagocytosis of Mφ is augmented in hypersplenism in portal hypertension; however, the specific mechanisms are not clear.

Research frontiers

The functions of macrophage are focused in the investigation of pathogenesis of hypersplenism in portal hypertension.

Innovations and breakthroughs

This study based on cDNA microarray has screened differentially expressed genes of macrophages between normal spleen and portal hypertensive spleen, which may provide a new idea in studying the pathogenesis of hypersplenism in portal hypertension.

Applications

Small interfering RNAs or other techniques may alter the expression of the differentially expressed genes, and may be used in treatment of hypersplenism in portal hypertension.

Peer review

The authors identified the difference in gene expression of Mφ between normal spleen and portal hypertensive spleen using cDNA microarrays, found that 121 genes were differentially expressed in all three chips, including 21 up-regulated known genes and 73 down-regulated known genes. The differentially expressed genes were related to the ionic channel and transport protein, cyclin, cytoskeleton, cell receptor, cell signal conduct, metabolism, immune, and so on. These genes might be also related to the hypersplenism in portal hypertension. These differentially expressed genes may provide some clues for further studies of hypersplenism in portal hypertension.

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RAPID COMMUNICATION

Anti-tumor activities and apoptosis-regulated mechanisms of bufalin on the orthotopic transplantation tumor model of human hepatocellular carcinoma in nude mice

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Abstract

AIM: To investigate anti-tumor activities and apoptosis-regulated mechanisms of bufalin in the orthotopic transplantation tumor model of human hepatocellular carcinoma in nude mice.

METHODS: BEL-7402 cells of human hepatocellular carcinoma were inoculated to form subcutaneous tumors, and were implanted into the liver to establish orthotopic transplantation tumor models of human hepatocellular carcinoma in nude mice. Seventy-five animals were randomized divided into five groups (n = 15). Bufalin was injected intraperitoneally into three groups at doses of 1.5 mg/kg (BF1), 1 mg/kg (BF2) and 0.5 mg/kg (BF3) for d 15-24, respectively. The NS group was injected an equal volume of saline as above and adriamycin was injected intraperitoneally into the ADM group at a dose of 8.0 mg/kg for d 15. Ten mice in each group were killed at d 25 and the survival time in each group was calculated. We also observed the morphologic alterations in the myocardium, brain, liver, kidney and tumor tissues by pathology and electron microscopy, measured the apoptotic rate by TUNEL staining method, and detected the expression of apoptosis-regulated genes bcl-2 and bax by immunohistochemical staining and RT-PCR in tumor tissues.

RESULTS: The tumor volumes in each group of bufalin were reduced significantly (35.21 ± 12.51 *vs* 170.39 ± 25.29; 49.83 ± 11.46 *vs* 170.39 ± 25.29; 83.99 ± 24.63 *vs* 170.39 ± 25.29, $P < 0.01$, respectively), and the survival times were prolonged in group BF1-2 (31.8 ± 4.2 *vs* 23.4 ± 2.1 and 29.4 ± 3.4 *vs* 23.4 ± 2.1, $P < 0.05$, respectively), and necrosis was mainly in severe or moderate degree in group BF1-2. No morphological changes were detected in the myocardium, brain, liver and kidney tissues. Apoptotic characteristics could be seen in group BF1-2. The positive rates of bcl-2 and bax protein expression of each group by immunohistochemical staining were 10.0%, 10.0%, 20.0%, 10.0% and 20.0%; 90.0%, 80.0%, 80.0%, 40.0% and 30.0%, respectively. Loss of expression of bcl-2 mRNA in each group was to be found and the density of bax mRNA was increased progressively with increase of dose of bufalin by RT-PCR.

CONCLUSION: Bufalin has significant anti-tumor activities in the orthotopic transplantation tumor model of human hepatocellular carcinoma in nude mice with no marked toxicity and was able to induce apoptosis of transplanted tumor cells. This apoptosis may be mediated mainly *via* up-regulating the expression of apoptosis-regulated gene bax, which may be involved in its anti-tumor mechanism of bufalin.

Key words: Bufalin; Hepatocellular carcinoma; Orthotopic transplantation; Nude mice; Model; Treatment; Apoptosis

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INTRODUCTION

Apoptosis occurs in several pathological situations in multicellular organisms and constitutes part of a common mechanism of cell replacement, tissue remodeling, and removal of damaged cells. Bcl-2 family plays a crucial role in the control of apoptosis and can be classified into two functionally distinct groups: antiapoptotic proteins and pro-apoptotic proteins. Bcl-2, an antiapoptotic protein, is known to regulate apoptotic pathways and protects against cell death. Bax, a pro-apoptotic protein of that family, is expressed abundantly and selectively during apoptosis and promotes cell death. Increasing the ratio of bcl-2 to bax has commonly been used to determine the induction of

apoptosis in several tissues[1,2].

Bufalinfalin (Bufalin) is a toxic ligand extracted from a traditional Chinese medicine *Secretia bufonis*, the molecular formula of which is $C_{24}H_{34}0_4$ with a relative molecular weight 386.5. Bufalin has the activities of inducing differentiation and apoptosis of tumor cells. Research on bufalin mainly involves tumor spectra of leukemia, prostate cancer, gastric cancer and liver cancer, and is confined to *in vitro* studies[3-11]. The results of our previous studies[12] have showed that a μmol dose of bufalin was able to produce a potent killing effect on human liver cancer cells *in vitro* within 48 h.

The present study was to use a human hepatocellular carcinoma *in situ* transplantation model of nude mice to observe the anti-tumor activities of bufalin *in vivo* and investigate the relation between this apoptosis and expression of bcl-2 and bax and to provide the theoretical and methodological basis for its clinical application in the future.

MATERIALS AND METHODS

Experimental animals and strains

Eighty male BALB/Cnu/nu nude mice (aged 4-5 wk and weighing 18-20 g) were provided by the experimental animal center of Fudan University. The nude mice were caged individually under specific-pathogen free (SPF) conditions. The code number of the animals was SCKK (Shanghai) 2004-0010. Eighteen male ICR mice (aged 4 wk and weighing 10-22 g) were provided by the experimental animal center of the Second Military Medical University. Human hepatocellular carcinoma cell strain BEL-7402 (Shanghai Institute of Cell Biology of the Chinese Academy of Sciences) was generation cryopreserved in our laboratory.

Drugs

Bufalin (10.0 mg/via) was purchased from American Sigma, and prepared to a 0.2 mg/mL concentration by adding 0.5 mL anhydrous alcohol and injection water. Adriamycin (ADM) (Wan Le Pharmaceuticals, Shenzhen, Batch No. 0505E1) was prepared into a 1.0 mg/mL concentration using normal saline.

Establishment of the in situ transplantation tumor model

The model was established by using the intrahepatic tunnel implantation[13]. Cryopreserved human liver cancer cell strain BEL-7402 was thawed, cultured *in vitro* to the log growth phase, centrifuged and washed with PBS to prepare to a concentration of 1×10^7 cells/mL. Each nude mouse was inoculated with 0.2 mL of the strain subcutaneously via the back. When the tumor grew to 1.0 cm in diameter, the fish meat-like fresh tumor tissue was cut into 1.0 mm × 2.0 mm pieces and implanted into the tunnel under the capsule of the left lateral liver lobe by using a pair of ophthalmologic forceps. The wound was slight pressed with a cotton swab for hemostasis and closed.

Toxicity test

Eighteen ICR mice were equally divided into 3 dose groups: 2.0 mg/kg, 1.5 mg/kg and 1.0 mg/kg bufalin intraperitoneally daily for 10 d. Weight, appetite and behavior of the animals were observed. Anti-tumor dose of bufalin was initially defined as 1.5 mg/kg, 1.0 mg/kg and 0.5 mg/kg.

Grouping and management of the model

Exploratory laparotomy was performed in all animals 14 d after establishment of the model; the model success rate was 100%. The 75 models were equally randomized into five groups: the large-, middle- and small-dose groups (group BF 1-3), the positive group (ADM group) and the negative group (NS group). Drug administration was initiated at 15 d after establishment of the model. Group BF 1-3 was administered the drug according to the dose groups of the toxicity test intraperitoneally daily from 15 to 24 d. The NS group received the equivalent amounts of normal saline in the same way. The ADM group received 8.0 mg/kg intraperitoneally at d 15. At d 25, 10 of the 15 animals in each group were sacrificed. Blood sampling from the eye ball was performed to test liver and kidney functions and blood routine. Volume of the tumors was measured. Tumor tissue, heart, liver, lung and kidney specimens were taken for routine pathology and electron microscopy observation. The remaining 5 animals in each group were kept alive for observation of tumor-bearing survival.

Indexes and method of observation

Tumor volume and tumor inhibitory rate: The longest diameter (a) and shortest diameter (b) of the tumor body were measured by using a slide gaud, and the tumor volume was calculated according to the formula $V = ab^2/2$; the tumor inhibitory rate = (1-mean tumor volume of the drug group/mean tumor volume of the control group) × 100%; prolonged survival = (mean days of survival of the drug group/mean days of survival of the control group-1) × 100%. The survival time was defined as the day from administering drugs to death. Histological examination: The remaining tissue of each group was formalin fixed, paraffin sectioned and HE stained for routine pathology to observe any change in morphology and tumor necrosis. Electron microscopic treatment: The tumor tissue was fixed with 3% glutaral for 1-3 h, washed with buffer solution, fixed with 1% osmium tetroxide for 1-2 h, gradient dehydrated with acetone, Epon812 embedded, uranyl acetate and lemon lead double stained, sliced, and then stained for transmission electron microscopy observation.

TUNEL assay

Tumor samples were cryopreserved in liquid nitrogen and cut into 8-μm thick slices. Slices were fixed in ice-cold 80% ethanol for 24 h, treated with proteinase K and 0.3% H_2O_2, labeled with fluorescein dUTP in a humid box for 1 h at 37℃. Slices were then combined with POD-horseradish peroxidase, stained with DAB and counterstained with methyl green. Controls received the same management except the labeling fluorescein dUTP. Cells were visualized with a light microscope. The apoptotic index (AI) was

calculated as follows: AI = number of apoptotic cells/total number × 100%.

Immunohistochemical staining

Tumor samples were cryopreserved in liquid nitrogen, cut into 8-μm thick slices and fixed by acetone. After washing with PBS, slices were incubated in 0.3% H_2O_2 solution at room temperature for 5 min. Slices were then incubated with anti-bcl-2 or anti-bax monoclonal antibody (purchased from Shanghai Biotechnology Co. Ltd) at a 1:300 dilution at 4℃ overnight. After washed with PBS, the second antibody, biotinylated antirat IgG, was added and cells were incubated at room temperature for 1 h. After washing with PBS, ABC compound was added and incubated at room temperature for 10 min. DAB was used as the chromagen. After 10 min, the brown color signifying the presence of antigens bound to antibodies was detected by light microscopy. Controls were managed as the experimental group except for the incubation of primary antibody. The positive criterion was defined as the positive rate must exceed 5%, and the positive rate (PR) was calculated as follows: PR = (number of positive cells/total number) × 100%.

RT-PCR

Tumor samples were cryopreserved in liquid nitrogen and total RNA was extracted. Concentration of RNA was determined by the absorption at 260 nm. The primers for bcl-2 mRNA, bax mRNA and β-actin were as follows: β-actin (587 bp) 5' CCAAGGCCAACCGCGAGAAGATG 3' (sense); 5' AGCGTACATGGTGG -TGCCGCCA 3' (antisense); bcl-2αmRNA (266 bp) 5' CCGAGATGTCCA-GCCAGCT 3' (sense); 5' CAGTTCCACAAAGGCATCC 3' (anti-sense); bcl-2βmRNA (727 bp) 5' TACGACAACCGGGACATAGTG 3' (sense); 5' GAACGCTTTGTCCAGAGGAG 3' (anti-sense) bax mRNA (292bp) 5' CGAGTGGCAGTGACATGT 3' (sense), 5' TCTTCTTCCAGATGGTGAG 3' (anti-sense). Polymerase chain reactions were performed in a 50 μL reaction volume. RT-PCR reaction was run under the following conditions: at 94℃ for 1 min, 1 circle; at 94℃ for 30 s, at 54℃ for 40 s, at 72℃ for 1 min, 32 circles; at 72℃ for 5 min, 1 circle. 15 μL PCR products were placed onto a 15 g/L agarose gel and observed by EB staining using the Gel-Pro analyzer.

Statistical analysis

Data analysis of variance was performed by SPSS 11.0 and $P < 0.05$ was considered statistically significant.

RESULTS

Primary toxicity test

Transient convulsion was observed 5 min after the first IP drug administration in half the animals of the 2.0 mg/kg bufalin groups, short-term listlessness was observed after the second injection, which was restored to normal 3-5 min later. No evident adverse reaction was observed in the 1.5 mg/kg and less groups. No evident change was observed in weight, appetite and behavior after 10 d of drug administration. There was no treatment-related death.

Table 1 Tumor volume, weight and tumor inhibitory rate pre- and post-treatment (mean ± SD, n = 10)

Group	Tumor volume (cm^3)		Tumor inhibitory rate	weight (g)	
	Pre-treatment	Post-treatment	(%)	Pre-treatment	Post-treatment
BF1	43.50 ± 5.09	35.21 ± 12.51[a,c]	79.3	20.94 ± 1.00	21.39 ± 1.62[e]
BF2	42.92 ± 4.10	49.83 ± 11.46[a,c]	70.7	20.03 ± 1.16	21.48 ± 1.10[e]
BF3	43.00 ± 2.97	83.99 ± 24.63[a]	50.7	21.06 ± 0.94	21.57 ± 1.14[e]
ADM	42.93 ± 4.23	55.17 ± 16.13[a]	67.6	21.00 ± 1.00	18.90 ± 0.77
NS	43.37 ± 4.82	170.39 ± 25.29	-	20.95 ± 1.07	20.40 ± 1.23

[a]$P < 0.001$ *vs* NS group; [c]$P < 0.05$ *vs* ADM group; [e]$P < 0.05$ *vs* ADM group.

Weight of tumor bearing mice and tumor volume pre- and post-treatment

Table 1 shows that there was no significant difference in tumor volume between these five groups ($P > 0.05$), indicating that the tumor volumes before treatment were comparable between these five groups. Compared with the NS group, the tumors of the Bufalin and ADM groups shrank significantly ($P < 0.01$), especially in the BF1 group with a tumor inhibitory rate of 79.3%. The tumor inhibitory rate of the BF2 group, ADM and BF3 group was 70.7%, 67.6% and 50.7%, respectively. The difference between the ADM group and BF1 group and 2 was significant ($P < 0.05$). There was no significant difference in weight between the other groups and the NS group ($P > 0.05$). Compared with the BF1-3 groups, the weight of the ADM group decreased significantly ($P < 0.05$).

Mean survival and life prolonging rate after treatment

Tumor bearing survival of bufalin groups was prolonged, and the difference was significant between group BF1-2 and NS and ADM groups ($P < 0.05$), especially in group BF1. Survival of ADM group was not significantly prolonged as compared with the NS group ($P > 0.05$) (Table 2).

Observation of tumor necrosis

The *in situ* transplanted tumors were relatively regular, shaping round or oval with complete or incomplete capsules, mainly in the form of infiltrating growth (Figure 1). HE stain revealed defuse tumor tissue with rich blood vessels in the interstitial and infiltrative liver tissue around the edge of the tumor. The nucleus of the liver cancer cell was large oval-shaped; there was more chromatin; binucleolates were large and clearly visible; there were more free ribosomes and more pathological karyokinesis (Figure 2A). Severe necrosis was mainly seen in the BF1-2 and ADM group, and mild necrosis in the NS group (Figure 2B). There was less cytoplasm in tumor cells of group BF1-3, where cell apoptotic signs were seen, such as nuclei became pyknotic, chromatin was concentrated and collected around the edge, and nuclei were cleft.

Ultrastructural change of tumor cells

Transmission electron microscopy revealed that in the NS group the surface of the tumor cells was irregular; there was more chromatin; large and clear binucleolates were frequently seen; there was an increase in free ribosomes;

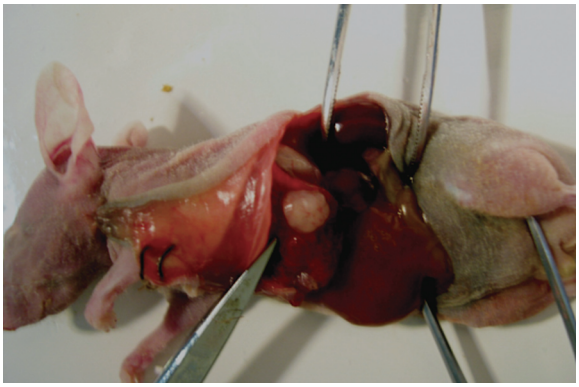

Figure 1 The orthotopic transplantation tumor model of human hepatocellular carcinoma in nude mice on 14 d, tumor size is 0.5 cm × 0.5 cm.

Table 2 Mean survival and life prolonging rate of each group (mean ± SD)

Group	*n*	Mean survival time (d)	Life prolonging rate (%)
BF1	5	31.8 ± 4.2[a,c]	35.9
BF2	5	29.4 ± 3.4[a,c]	25.6
BF3	5	28.4 ± 3.9[c]	31.8
ADM	5	22.2 ± 1.6	21.4
NS	5	23.4 ± 2.1	-

[a]$P < 0.05$ *vs* NS group; [c]$P < 0.05$ *vs* ADM group.

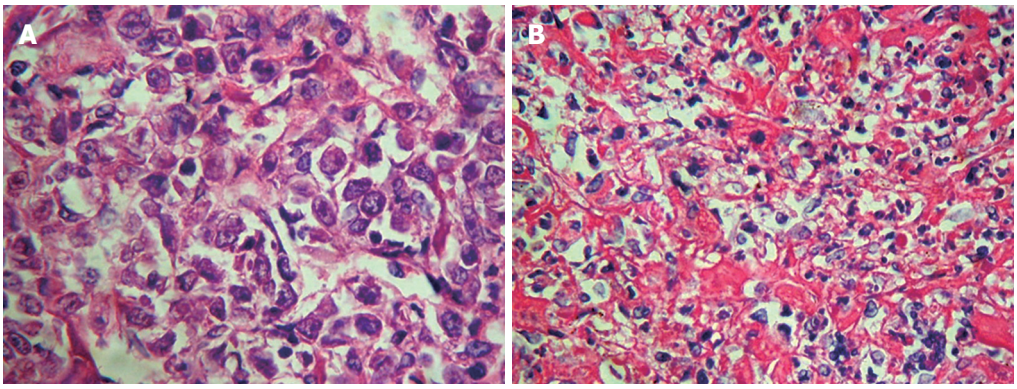

Figure 2 **A**: Pathological changes of the orthotopic transplantation tumor cells of NS group in nude mice (× 200); **B**: Pathological changes of the orthotopic transplantation tumor of BF1 group in nude mice (× 100).

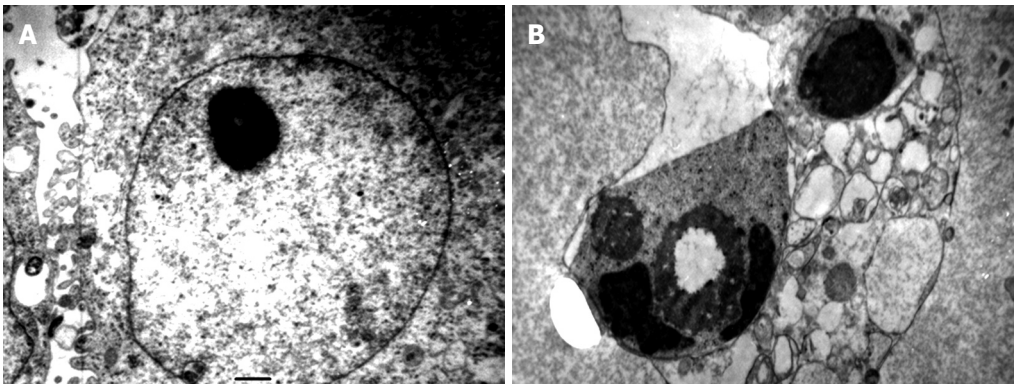

Figure 3 **A**: Ultrastructural changes of the orthotopic transplantation tumor of NS group in nude mice (scanning electron microscope × 10 000); **B**: Characteristic apoptosis in the orthotopic transplantation tumor cells of BF1 group in nude mice (scanning electron microscope × 10 000).

rich mitochondrion and endoplasmic reticulum were seen in the cytoplasm (Figure 3A). In BF groups, cell shrinkage, cytoplasm concentration, collection of concentrated chromatin on the medial side of the nuclear membrane in the form of masses or crescents with bubbling of cytoplasm and apoptotic corpuscles (Figure 3B).

TUNEL assay

Positive staining was located in the nuclei (Figure 4). The apoptosis index of group BF1-3, ADM and NS group was 10.60% ± 3.42%, 8.86% ± 2.96%, 5.87% ± 2.13%, 4.26% ± 2.12% and 3.28% ± 0.98%, respectively ($P < 0.01$ or $P < 0.05$, *vs* the NS group).

Expression of bcl-2 and bax protein

Positive staining was located in the cytoplasm (Figure 5A and B). The positive rates of bcl-2 and bax protein of group BF 1-3, ADM and NS group was 10.0%, 10.0%, 20.0%, 10.0% and 20.0%; 90.0%, 80.0%, 80.0%, 40.0% and 30.0% respectively by immunohistochemical staining ($P > 0.05$, *vs* the NS group; $P < 0.01$ or $P < 0.05$, *vs* the NS group, Table 3).

RT-PCR

Loss of expression of bcl-2 mRNA in each group was to be found and the density of bax mRNA was increased progressively with the increase of dose of bufalin by RT-PCR (Figure 6A and B).

DISCUSSION

Until now, few chemotherapeutic drugs are effective

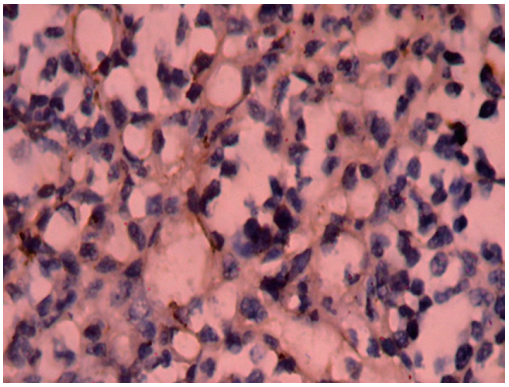

Figure 4 Apoptosis in the orthotopic transplantation tumor cells guided by bufalin in nude mice (TUNEL × 100).

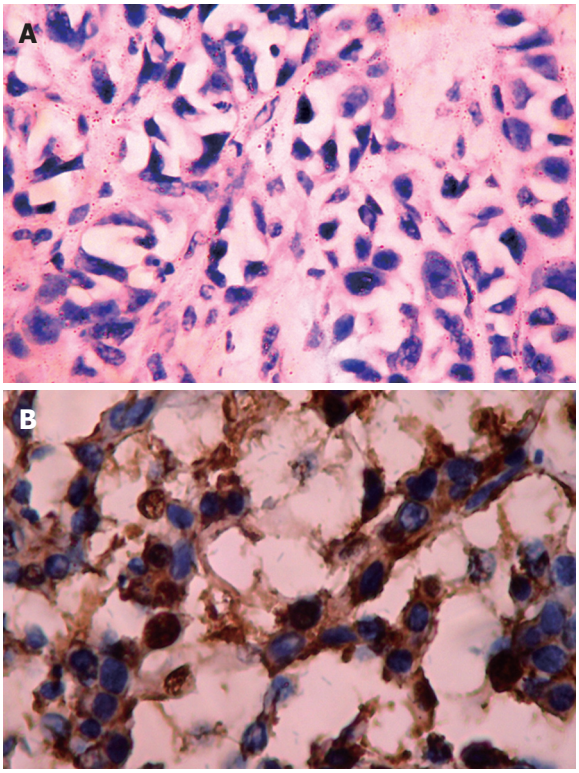

Figure 5 **A**: Nagative expression of bcl-2 in the orthotopic transplantation tumor group of nude mice (× 200); **B**: Positive expression of bax in the orthotopic transplantation tumor group of nude mice (× 200).

Table 3 The expression of bcl-2 or bax protein of tumor tissues in nude mice (mean ± SD, n = 10)

Group	bcl-2		Positive rate (%)	*P*	bax		Positive rate (%)	*P*
	-	+			-	+		
BF1	9	1	10	> 0.05	1	9	90[a]	< 0.01
BF2	9	1	10	> 0.05	2	8	80[c]	< 0.05
BF3	8	2	20	> 0.05	2	8	80[c]	< 0.05
ADM	9	1	10	> 0.05	6	4	40	> 0.05
NS	8	2	20	-	7	3	30	-

[a]$P < 0.01$ *vs* NS group; [c]$P < 0.05$ *vs* NS group.

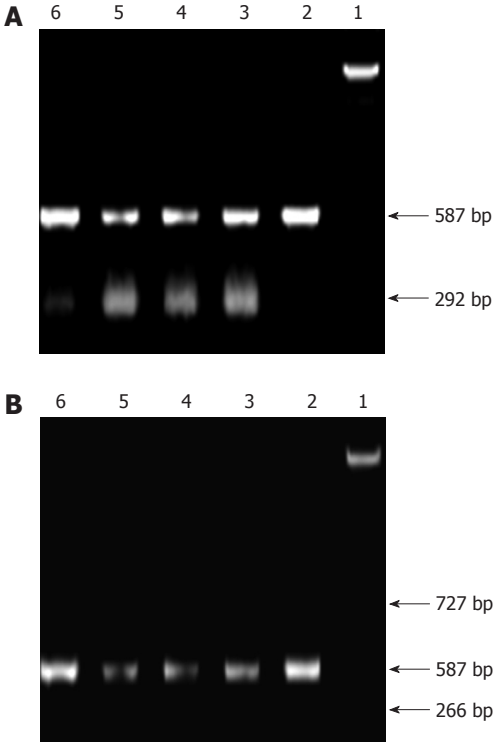

Figure 6 Expression of bax mRNA (**A**) and bcl-2 mRNA (**B**) in apoptotic transplanted tumor cells induced by bufalin.

in the treatment of human hepatocellular carcinoma and it is necessary to look for new anti-hepatocellular carcinoma drugs. Bufalin is a toxic traditional Chinese medicine, mainly containing resibufogenin, cinobufagin, cinobufotalin, bufotalin and bufalin, of which bufalin has the strongest anti-tumor activities. Our previous studies showed that bufalin had marked anti-tumor activities on the human liver cancer cell lines SMMC-7721 and BEL-7402[12], on the basis of which the present study used the nude mice human liver cancer *in situ* transplantation model to study the anti-tumor activities of bufalin *in vivo*. The results show that tumor volumes were reduced significantly in group BF1-2 (as compared with the NS group, $P < 0.01$), tumor bearing survival was prolonged significantly (compared with the NS group, $P < 0.05$), and there was no significant change in weight ($P > 0.05$); tumor volumes of group BF1-2 were reduced significantly as compared with the ADM group ($P < 0.05$), and survival of the animals in the ADM group was not significantly prolonged (compared with NS group, $P > 0.05$) where weight of the animals decreased significantly (compared with the other groups, $P < 0.05$). Light electron microscopy showed moderate and severe necrosis of tumor tissues in group BF1-2, and moderate and mild necrosis in group BF1. No morphological changes were found in the heart, brain, liver, kidney and lung tissues; liver and kidney functions and blood were not significantly affected (data not shown). Transmission electron microscopy revealed more apoptotic changes of tumor tissues in group BF1-2. We assume that this is the result of the anti-tumor activities of bufalin.

In studies using 10 μmol bufalin of the above concentrations to treat HL-60, ML-1 and U937 cell lines, Jing

et al[14] found morphological changes seen in apoptotic cells, which included cell shrinkage, chromatin aggregation, cleavage, and formation of apoptotic corpuscles. No cell apoptosis was observed in the experiment using 1 μmol/L bufalin to treat normal human monocytes and polymorphic nuclear cells for 24 h. These results indicate that the actions of bufalin in selectively inhibiting tumor growth and inducing cell apoptosis are closely related. The results of the present study demonstrated that bufalin has the action of inducing apoptosis of *in situ* human liver cancer cells.

The Bcl-2 family plays a crucial role in the control of apoptosis. It has been found that the family includes a number of proteins which have homologous amino acid sequences, including antiapoptotic members such as bcl-2 and bcl-xL, as well as proapoptotic members including bax and bad[15,16]. Over expression of bax could promote cell death[1,2,17]. Conversely, over expression of antiapoptotic proteins such as Bcl-2 could repress the function of bax[18,19]. Thus, the ratio of bcl-2 /bax was a critical determinant of a cell's threshold for undergoing apoptosis[20]. In this study, we evaluated the effectiveness of apoptosis *in situ* human liver cancer cells induced by bufalin *in vivo*, this apoptosis might be mediated by up-regulating the expression of apoptosis-regulated gene bax and decreased the ratio of bcl-2/bax of human liver cancer cells.

It is reported in the literature[21] that ID_{50} of bufalin is 2.2 mg/kg. Our pre-experiment also showed that 2.0 mg/kg bufalin caused spasmodic seizures but did not cause toxic death in mice. The results suggest that 1.5 mg/kg bufalin would prove to be the appropriate anti-tumor dose. The most appropriate dosage of bufalin for other tumors needs to be further observed.

The results of the present study showed that bufalin had marked anti-tumor activities and was able to induce apoptosis which might be mediated by up-regulating the expression of apoptosis-regulated gene bax and decreased the ratio of bcl-2/bax in the human *in situ* liver cancer transplantation model in nude mice with no evidence of toxicity to the heart, lungs, liver, kidneys and brain. Bufalin' s selective inhibition on tumor cell warrants further study with respect to its anti-tumor effect *in vivo*, the optimal dosage and the other related mechanisms.

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RAPID COMMUNICATION

Study of circumferential resection margin in patients with middle and lower rectal carcinoma

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Abstract

AIM: To clarify the relationship between circumferential resection margin status and local and distant recurrence as well as survival of patients with middle and lower rectal carcinoma. The relationship between circumferential resection margin status and clinicopathologic characteristics of middle and lower rectal carcinoma was also evaluated.

METHODS: Cancer specimens from 56 patients with middle and lower rectal carcinoma who received total mesorectal excision at the Department of General Surgery of Guangdong Provincial People's Hospital were studied. A large slice technique was used to detect mesorectal metastasis and evaluate circumferential resection margin status.

RESULTS: Local recurrence occurred in 12.5% (7 of 56 cases) of patients with middle and lower rectal carcinoma. Distant recurrence occurred in 25% (14 of 56 cases) of patients with middle and lower rectal carcinoma. Twelve patients (21.4%) had positive circumferential resection margin. Local recurrence rate of patients with positive circumferential resection margin was 33.3% (4/12), whereas it was 6.8% (3/44) in those with negative circumferential resection margin (P = 0.014). Distant recurrence was observed in 50% (6/12) of patients with positive circumferential resection margin; conversely, it was 18.2% (8/44) in those with negative circumferential resection margin (P = 0.024). Kaplan-Meier survival analysis showed significant improvements in median survival (32.2 ± 4.1 mo, 95% CI: 24.1-40.4 mo *vs* 23.0 ± 3.5 mo, 95% CI: 16.2-29.8 mo) for circumferential resection margin-negative patients over circumferential resection margin-positive patients (log-rank, $P < 0.05$). 37% T_3 tumors examined were positive for circumferential resection margin, while only 0% T_1 tumors and 8.7% T_2 tumors were examined as circumferential resection margin. The difference between these three groups was statistically significant (P = 0.021). In 18 cancer specimens with tumor diameter ⩾ 5 cm 7 (38.9%) were detected as positive circumferential resection margin, while in 38 cancer specimens with a tumor diameter of < 5 cm only 5 (13.2%) were positive for circumferential resection margin (P = 0.028).

CONCLUSION: Our findings indicate that circumferential resection margin involvement is significantly associated with depth of tumor invasion and tumor diameter. The circumferential resection margin status is an important predictor of local and distant recurrence as well as survival of patients with middle and lower rectal carcinoma.

Key words: Middle and lower rectal carcinoma; Circumferential resection margin; Prognosis

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INTRODUCTION

It is well known that middle and lower rectal carcinomas are of the most common carcinomas in China. However, even after undergoing radical resection of primary tumors and lymph nodes, about 5%-40% of patients with rectal carcinoma report with local recurrence[1-5]. It has been reported that residual mesorectal metastasis may be the most important factor of local recurrence[6,7]. In the current study, circumferential resection margin was detected by pathological observation in cancer specimens from 56 patients with middle and lower rectal carcinoma. The associations of circumferential resection margin status and local recurrence, distant recurrence as

well as clinicopathologic characteristics of patients with middle and lower rectal carcinoma were investigated. The relationships between circumferential resection margin status and clinicopathologic characteristics of middle and lower rectal carcinoma were also evaluated.

MATERIALS AND METHODS

Patients

Cancer specimens resected from 56 patients with middle and lower rectal carcinoma who received total mesorectal excision at the Department of General Surgery of Guangdong Provincial People's Hospital from November 2001 to July 2003 were studied. There were 37 men and 19 women, ranging in age from 30 to 86 years, with a mean age of 60.5 years. None of these patients had received preoperative chemotherapy or radiotherapy. There were 26 lower rectal carcinomas and 30 middle rectal carcinomas. Patients with tumor diameter ⩾ 5 cm were in 18 cases, with tumor diameter < 5 cm in 38 cases. Low anterior resection was performed in 40 patients, abdominal perineal resection in 16 patients. According to the Ming's criteria, 15 tumors were classified as expansive type carcinomas, 41 tumors classified as infiltrative type carcinomas. TNM stage status: stage I in 5 patients, stage Ⅱ in 22 patients, and stage Ⅲ in 29 patients. There were 14 patients with poorly differentiated carcinoma, 37 patients with moderately differentiated carcinoma, and 5 patients with well-differentiated carcinoma.

Methods

Two pathologists who had no knowledge of the clinicopathological data observed the specimens independently. If tumor cells were detected within 1 mm of the circumferential margin, the status was classified as positive circumferential resection margin[8].

Statistical analysis

Statistical analysis was performed by the Pearson Chi-square test to examine the associations of circumferential resection margin status and local recurrence, distant recurrence as well as clinicopathologic characteristics of patients with middle and lower rectal carcinoma. The relationship between circumferential resection margin status and survival of patients with middle and lower rectal carcinoma was evaluated by Kaplan-Meier survival analysis and log-rank test. Statistical significance was defined as $P < 0.05$.

RESULTS

Correlations between circumferential resection margin status and local recurrence, distant recurrence as well as survival of patients with middle and lower rectal carcinoma

Local recurrence occurred in 12.5% (7 of 56 cases) of patients with middle and lower rectal carcinoma. Distant recurrence occurred in 25% (14 of 56 cases) of patients with middle and lower rectal carcinoma. Twelve patients (21.4%) had positive circumferential resection margin. Local recurrence rate of patients with positive circumferential resection margin was 33.3% (4/12), whereas it was 6.8% (3/44) in those with negative circumferential resection margin ($P = 0.014$). Distant recurrence was observed in 50% (6/12) of patients with positive circumferential resection margin; conversely, it was 18.2% (8/44) in those with negative circumferential resection margin ($P = 0.024$). Kaplan-Meier survival analysis showed significant improvements in median survival (32.2 ± 4.1 mo, 95% CI: 24.1-40.4 mo *vs* 23.0 ± 3.5 mo, 95% CI: 16.2-29.8 mo) for circumferential resection margin-negative patients over circumferential resection margin-positive patients (log-rank, $P < 0.05$) (Figure 1).

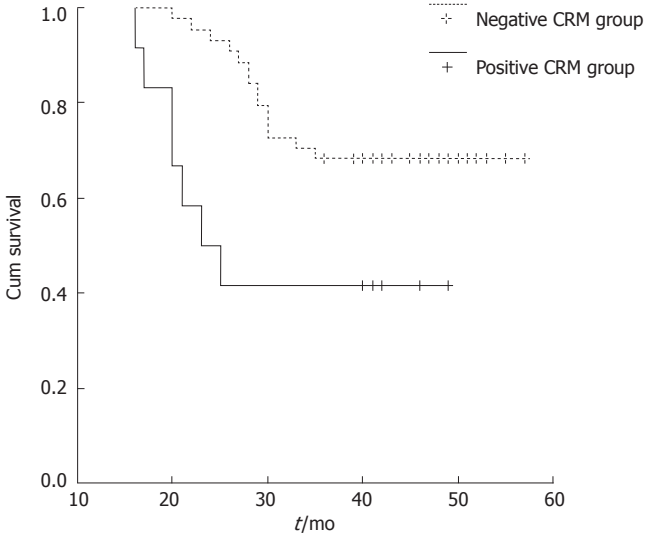

Figure 1 Correlation between circumferential resection margin status and survival of patients with middle and lower rectal carcinoma (Kaplan-Meier survival analysis).

Correlations between circumferential resection margin status and clinicopathologic characteristics of patients with middle and lower rectal carcinoma

The circumferential resection margin involvement correlated significantly with depth of tumor invasion and tumor diameter. 37% T_3 tumors were examined as positive circumferential resection margin, while only 0% T_1 tumors and 8.7% T_2 tumors were examined as circumferential resection margin. The difference between these three groups was statistically significant ($P = 0.021$). In 18 cancer specimens with tumor diameter ⩾ 5 cm 7 (38.9%) were detected positive circumferential resection margin, while in 38 cancer specimens with tumor diameter < 5 cm only 5 (13.2%) were positive circumferential resection margin ($P = 0.028$). No significant correlations were found between circumferential resection margin involvement and other variables such as age ($P = 0.815$), gender ($P = 0.961$), diameter of tumor infiltration ($P = 0.417$), tumor differentiation ($P = 0.074$), Ming's classification ($P = 0.372$) and lymph node metastases ($P = 0.609$) (Table 1).

DISCUSSION

After radical resection of rectal carcinoma, the circumferential resection margin on the non-peritonealized surface of the resected specimen is of critical importance.

Table 1 Correlation between circumferential resection margin involvement with clinicopathologic characteristics of patients with middle and lower rectal carcinoma

Variable	*n*	Circumferential resection margin involvement		
		Negative (%)	Positive (%)	*P*
Gender				
Male	37	29 (78.4)	8 (21.6)	
Female	19	15 (78.9)	4 (21.1)	$P = 0.961$
Age (yr)				
< 60	25	20 (80.0)	5 (20.0)	
⩾ 60	31	24 (77.4)	7 (22.6)	$P = 0.815$
Superficial diameter (cm)				
< 5	38	33 (86.8)	5 (13.2)	
⩾ 5	18	11 (61.1)	7 (38.9)	$P = 0.028$
Diameter of infiltration				
1/4	8	7 (87.5)	1 (12.5)	
1/2	16	14 (87.5)	2 (12.5)	
3/4	18	14 (77.8)	4 (22.2)	
4/4	14	9 (64.3)	5 (35.7)	$P = 0.417$
Ming's classification				
Expansive	15	2 (13.3)	13 (86.7)	
Infiltrative	41	10 (24.4)	31 (75.6)	$P = 0.372$
Depth of invasion				
T_1	6	6 (100.0)	0 (0)	
T_2	23	21 (91.3)	2 (8.7)	
T_3	27	17 (63.0)	10 (37.0)	$P = 0.021$
Histologic differentiation				
Well	5	4 (80.0)	1 (20.0)	
Moderate	37	32 (86.5)	5 (13.5)	
Poorly	14	8 (57.1)	6 (42.9)	$P = 0.074$
Lymph node metastasis				
Positive	29	22 (75.9)	7 (24.1)	
Negative	27	22 (81.5)	5 (18.5)	$P = 0.609$

7.1%-35% of patients with rectal carcinoma were reportedly identified circumferential resection margin involvement[3,8-10]. In the present study, circumferential resection margin involvement in patients with middle and lower rectal carcinoma who underwent radical resection and total mesorectal excision were evaluated. 21.4% (12 of 56 cases) of patients had positive circumferential resection margin.

The correlation between circumferential resection margin status and local recurrence of patients with rectal carcinoma is still controversial presently[10-13]. Wibe *et al*[13] reported that positive circumferential resection margin had a significant and major prognostic impact on the rates of local recurrence of patients with rectal carcinoma who underwent total mesorectal excision. After a median follow-up of 29 (range 14-60) mo, the overall local recurrence rate was 7% (46 of 686 patients): 22% among patients with a positive resection margin and 5% in those with a negative margin. However, Luna-Perez[10] *et al* reported that circumferential resection margin involvement was not correlated significantly with local recurrence of patients with rectal adenocarcinoma ($P = 0.33$). Hall *et al*[11] reported that local recurrence rate of patients with positive circumferential resection margin was 15%, whereas it was 11% in those with negative circumferential resection margin. The difference between these two groups was not statistically significant ($P = 0.38$). Our results demonstrated that circumferential resection margin involvement had significant correlation with local recurrence of patients with middle and low rectal carcinoma. Local recurrence was more frequent in patients with positive circumferential resection margin (4 of 12 cases, 33.3%), compared with patients with negative circumferential resection margin (3 of 44 cases, 6.8%) ($P = 0.014$). We conclude that the circumferential resection margin status is an important predictor of local recurrence of patients with middle and low rectal carcinoma.

We also found that circumferential resection margin involvement was significantly correlated with distant recurrence and survival of patients with middle and low rectal carcinoma. Distant recurrence was observed in 50% (6/12) of patients with positive circumferential resection margin; conversely, it was 18.2% (8/44) in those with negative circumferential resection margin ($P = 0.024$). Kaplan-Meier survival analysis showed significant improvements in median survival for circumferential resection margin-negative patients over circumferential resection margin-positive patients (log-rank, $P < 0.05$). The consequences indicate that the circumferential resection margin status is an important predictor of local and distant recurrence as well as survival. For this reason, the circumferential resection margin status should be considered a major prognostic factor and should be validated in future trials as an early alternative clinical endpoint. Our results also support that histopathological examination of resected specimens must include careful assessment of the circumferential resection margin.

Many clinical studies reported the existence of circumferential resection margin involvement in patients with rectal carcinoma[8-15]. However, the relationships between circumferential resection margin status and clinicopathologic characteristics have not yet been explored. Therefore, the main objective of this study was to examine circumferential resection margin involvement and explore its relationship with clinicopathologic characteristics of patients with middle and lower rectal carcinoma. The circumferential resection margin involvement correlated significantly with depth of tumor invasion and tumor diameter. Positive circumferential resection margin was more frequent in T_3 tumors (20 of 27 cases, 37%), compared with T_2 tumors (2 of 23 cases, 8.7%) and T_1 tumors (0 of 6 cases, 0%). The difference between these three groups was statistically significant ($P = 0.021$). 38.9 per cent (7 of 18 cases) of patients with tumor diameter ⩾ 5 cm were detected as positive circumferential resection margin, while only 13.2% (5 of 38 cases) of patients with tumor diameter < 5 cm had circumferential resection margin involvement ($P = 0.028$). The result indicates that wider mesorectal excision should be followed in the management of patients with T_3 tumors or tumor diameter ⩾ 5 cm.

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CASE REPORT

Multiple gastrointestinal stromal tumors and bilateral pheochromocytoma in neurofibromatosis

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Abstract

The coincidence of a gastrointestinal stromal tumor (GIST) and a neuroendocrine tumor (NET) in neurofibromatosis type 1 (NF1) is described only five times within the literature. We report on a 63 year old Caucasian female with the rare condition of neurofibromatosis type 1 coinciding with recurrent gastrointestinal stromal tumor plus bilateral pheochromocytoma (PCC). After a history of palpitations and dizziness that lasted for years, a left adrenal mass was detected by CT. Laparotomy revealed a pheochromocytoma of the left adrenal gland while an ileoterminal GIST was found incidentally intraoperatively. After six months contralateral PCC and multiple recurrent GIST were resected again. After four years the patient is doing well without any signs of further recurrent tumors. Discussion includes review of the literature.

Key words: Gastrointestinal stromal tumor; Neuroendocrine tumor; Neurofibromatosis; Pheochromocytoma; Coincidence

Kramer K, Hasel C, Aschoff AJ, Henne-Bruns D, Wuerl P. Multiple gastrointestinal stromal tumors and bilateral pheochromocytoma in neurofibromatosis. *World J Gastroenterol* 2007; 13(24): 3384-3387

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INTRODUCTION

Incidence of neuroendocrine tumors (NET) and gastrointestinal stromal tumors (GIST) is 0.5 and 1-2 respectively in 100 000 persons per year. Prevalence of neurofibromatosis type 1 (NF1) is 1 in 3000 live births in Western Countries[1-3]. We report on a case with the rare condition of neurofibromatosis Type1 (von Recklinghausen) coinciding with GIST plus bilaterally pheochromocytoma (PCC).

CASE REPORT

A 63-year-old Caucasian female with NF1-was presenting with a history of recurrent palpitation and hypertension over 13 years associated with nausea and headache. There was no family history of NF1. Recurrent diagnostic check ups-(including specific cardiac and neurological consultation)-did not explain the symptoms. Finally a CT scan confirmed a tumor of the left adrenal gland, 5 cm in diameter. Hormone analyses were nonspecific, with only VMA slightly increased. Surgical resection of the tumor including the left adrenal gland was performed (Figure 1) via transversal laparotomy. In addition, a gastrointestinal stromal tumor of 6.5 cm in diameter, incidentally found within the ileoterminal mesenterial region (Figure 2) was also completely resected. During preparation of the left adrenal gland a hypertensive crisis occurred resulting in hypotension after adrenalectomy. Both could be resolved by anesthesiological intensive care support. After the operation the patient was referred to the intensive care unit where she stayed for two more days.

Resected specimens

Left sided adrenal gland, 85 g in weight, 4.5 cm in diameter with a grey-tan colored cut surface including cystic changes with hemorrhage compared to the yellow cortex surrounding it and a small remnant of remaining adrenal. In addition a 8.5 cm segment of the ileum with a solid grayish tumor of 6.5 cm in diameter, which did not relate to the mucosal surface, was removed. Immunohistochemistry confirmed pheochromocytoma of the left adrenal gland with expression of the S100 protein; GIST of the ileoterminal mesenterial region was verified by expression of c-kit and CD34 but not S100 or sm-actin. MIB-1 staining showed a proliferative activity of 2% in tumor cells.

Six months after surgery, a routine control CT scan showed contra-laterally a reemerging tumor of the right adrenal gland and also a mass ventral of the left kidney (Figure 3). Recurrently the patient's blood pressure was up to 200 mm Hg, associated with headaches and on and off palpitations. Within six weeks the blood pressure was reduced to low normal values by pharmacological

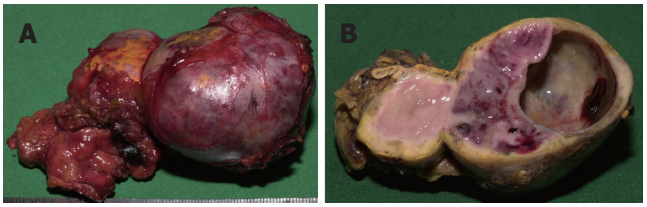

Figure 1 Adrenalectomy specimen from the left side containing a sharply circumscribed tumor (pheochromocytoma) of a x b cm in diameter. **A**: The intact resectate; **B**: A cross section with partial cystic transformation on the right and residual adrenal cortex at the periphery of the tumor.

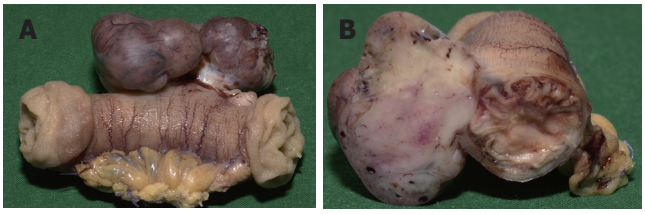

Figure 2 Segmental resectate of the small bowel with a lobulated subserosal tumor mass originating from the muscular bowel wall. **A**: The intact resection specimen; **B**: A cross section perpendicular to the bowel axis.

alpha blockage. During this time the size of both tumors persisted. Serum and urine levels of catecholamines and VMA remained normal. Finally a transversal relaparotomy was performed with enucleation of the tumor mass in the right adrenal gland while preserving its cortical part. Approximately 20 cm distal of the duodeno-jejunal ligament a jejunal tumor 3 cm in diameter was removed by resection of the jejunal segment. In addition, seven small jejunal tumors measuring up to 2 mm were also resected. The post operative follow up was inconspicuous without any complications.

Resected specimens of the second resection

subtotal resection of the right adrenal gland, revealing a pheochromocytoma measuring 4.5 cm in diameter as well as a solid, grayish-brown tumor of 2.8 cm in diameter in a segment of the ileum as well as 7 small tumor nodules with no relation to the mucosal surface confirmed as GIST by immunohistochemistry.

During follow ups 40 mo and 48 mo after the second operation, the patient feels well without any sign of neoplastic recurrence (CT) nor of any hyper-adrenergic symptoms.

BRIEF REVIEW OF THE LITERATURE: GIST AND NET IN NF1

Neurofibromatosis

NF1 is an autosomally inherited neurofibromatosis occurring with an incidence of about 1 per 3000 births, equally involving males and females. Genetically it is caused by a mutation at the NF1 gene located on chromosome 17q11.2[2,3]. Its gene product-neurofibromin-acts as a tumor suppressor. Functionally neurofibromin reduces cell

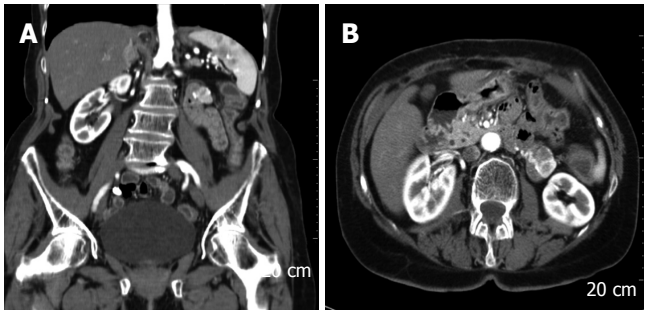

Figure 3 Computed tomography showing the right sided pheochromocytoma (**A**) and the jejunal GIST (**A** and **B**).

proliferation by accelerating the inactivation of the proto-oncogene p21-ras, which plays a cardinal role in mitogenic intracellular pathways[4,5]. Although genetic mutations have been described and the responsible gene product-neurofibromin-has been fully characterized, no frequently recurring mutation has been identified, and diagnosis is still based on established clinical criteria. The "classical triad" of symptoms are "café au lait" spots (CALs), cutaneous neurofibroma, and neoplasms of the peripheral or central nervous system. Malignancies are found in 3% to 15% of patients[2]. The frequencies of gastrointestinal complications in patients with NF1 disease are varying from 12% to 60% of cases and arise during midlife, later than cutaneous lesion[6-8]. They usually occur in three principal forms: hyperplasia of submucosal or myenteric nerve plexus, GIST and periampullary neuroendocrine tumors, sometimes associated with pheochromocytoma[6]. The clinical onset of these lesions, which mostly remain asymptomatic is in general characterized by abdominal pain, obstruction or by bleeding[2].

Epidemiology of GIST and NET in NF1: Only five similar cases with the coincidence of GIST and NET in NF1 have been published[9-13]. World wide in summary there are now six cases of neuroendocrine tumors associated with gastrointestinal stromal tumors and neurofibromatosis-as listed in Table 1[9-13]. It was not clear yet whether these coincidences are just accidental or if there might be a deep underlying relationship of these diseases (see below).

Epidemiology of GIST in NF1: Patients with NF1 have an increased risk of developing gastrointestinal tumors including rare types such as GIST[7] and GISTs are increasingly being recognized in association with NF1[14]. The incidence of GIST among NF1 patients is varying from 3.9% to 25%, while the overall ratio of NF1 in GIST patients counts up to 6% (10/167)[15,16].

Clinical characteristics of GIST in NF1: Initial clinical manifestation varies: unspecific abdominal pain, palpable abdominal mass, bowel obstruction and/or perforation with a high rate of incidental or emergency cases. Initial manifestation with gastrointestinal bleeding is common[7]. Depending on localization diagnostic procedures include upper and lower endoscopy, CT and MRI scan[17]. GIST in NF1 predominantly involving the small intestine including the duodenum, while in two thirds of the patients - like in the presented case - the tumors often occur in multiples[15,16].

Table 1 Review of the literature. Six cases with the coincidence of NET and GIST in Neurofibromatosis type 1 (NF1)

Patient age, sex	GIST Location / Diameter	NET Location + Histology /Diameter	Surgery	Follow up / outcome	Reference [no]/ year of publication
36, m	Jejunum 3, 5 cm	Papilla of vater n.i. 1, 2 cm	Duodeno-pancreatectomy	Two years/ well, no recurrence	Karatzas *et al*[10] 2000
60, m	Duodenojejunal n.i.	Pheochromocytoma (bilateral) n.i.	n.i. (Article in Italian)	n.i.	Rizzo *et al*[9] 2001
64, f	Duodenum, Jejeunum, Stomach Numerous: 0.5-2 cm	Papilla of vater Somatostatinoma n.i.	Local resection	n.i.	Usui *et al*[11] 2002
43, f	Jejunum 3, 5 cm	Duodenum neuroendocrine carcinoma 2, 7 cm	Duodeno-pancreatectomy	54 mo/ well	Kramer *et al*[12] 2005
60, m	Small bowel numerous: cm	Pheochromocytoma (bilateral)	n.i.	n.i.	Lisewski *et al*[13] 2006
63, f	Ileum 6, 5 cm Jejunum (prerenal) 3 cm	Pheochromocytoma (metachron, bilateral) 5 cm, left adrenal gland right adrenal gland 3 cm	Adrenalectomy intestinal segment resection tumor resection jejunal resection	48 mo/ well, no recurrence	as presented here

n.i.: no information.

Table 2 Time follow up of the presented case

Time	Tumor (size)	Localization	Treatment
0	PCC (3 cm) GIST (6.5 cm)	Left adrenal gland ileo-terminal	Adrenalectomy segment resection
9 m	PCC (3 cm) GIST (3 cm) 7 x GIST (2 mm)	Right adrenal gland proximo-jejunal	Tumor-resection segment resection tumor resection
45 m	No tumor		Corticoid substitution

PCC: pheochromocytoma; GIST: gastrointestinal stromal tumor; m: month; cm: centimetre; mm: millimetre.

Histology of GIST in NF1: The cross sectional imaging appearance of GIST tumors that occur in patients with NF1 is similar to that of gastrointestinal stromal tumors that occur in the general population[15]. The majority of these tumors are small and mitotically inactive; associated Cajal cell hyperplasia is common[16].

Pathogenesis of GIST in NF1: Ligand independent activation of c-kit (90%-95%) or alternatively of PGGFR-A (5%) is supposed to be the underlying pathogenetic mechanism of sporadic GIST. Different studies have shown that KIT and PDGFRA mutations are rarely found in GISTs in patients with NF1 suggesting that the pathogenesis of GIST in NF1 patients is different from that in non-NF1 patients[14-16]. Some authors presume that Kit germline mutation might be implicated in the pathogenesis of GIST at least in some NF1 patients[18]. Some authors discuss that in the pathogenesis of GIST in NF1 the loss of heterozygosity on the long arm of chromosome 22 (22q)-(allelic losses at 22q)-might be relevant because in 84% of these patients they are associated with high mitotic activity[9,10].

First published in 2004 by Kinoshita *et al*[19] and more recently confirmed by Maertens *et al* 2006[14] it is described that mutations in the NF1-gene might be involved in the pathogenesis of GIST in NF1 patients. In summary their demonstrated data suggest that (1) the NF1-related GISTs do not have KIT or PDGFRA mutations, (2) the molecular event underlying GIST development in this patient group is a somatic inactivation of the wild-type NF1 allele in the tumor and (3) inactivation of neurofibromin (NF1 gene) is an alternate mechanism to (hyper) activate the MAP-kinase pathway, while the JAK-STAT3 and PI3K-AKT pathways are less activated in NF1-related GIST compared with sporadic GISTs. Maertens *et al*[14] conclude, that was based on this molecular pathogenesis of GISTs in NF1 individuals that this type of tumor clearly belongs to the spectrum of clinical symptoms in NF1.

CONCLUSION

Predisposition of tumorgenesis in NF1 might be explained by overexpression of p21-ras a tumor-suppressor-gene called neurofibromin. Nevertheless the described concurrence of neuroendocrine tumor and gastrointestinal stromal tumor in NF1 is very rare (Table 2). In patients with von Recklinghausen neurofibromatosis the appearance of gastrointestinal symptoms should raise interest to search for gastrointestinal tumors because these patients are at risk for gastrointestinal neoplasms from which symptomatic patients are likely to experience significant morbidity. In accordance with other authors we also recommend that NF-1 patients with gastrointestinal symptoms receive further survey to rule out GISTs[14-16]. Further studies including molecular analysis to clarify the relationship between gastrointestinal tumors-in particular GIST-and neurofibromatosis are needed.

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CASE REPORT

Malignant lymphoma in the ileum diagnosed by double-balloon enteroscopy

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Abstract

A 73-year old man presented with abdominal pain. A tumor with central ulceration was observed in the ileum using double-balloon enteroscopy. Histological findings of the biopsy specimens were consistent with malignant lymphoma. Double-balloon enteroscopy confirmed the diagnosis of a malignant lymphoma tumor which was surgically resected. The patient is still in complete remission now.

Key words: Malignant lymphoma; Double-balloon enteroscopy

Beppu K, Osada T, Nagahara A, Sakamoto N, Shibuya T, Kawabe M, Terai T, Ohkusa T, Ogihara T, Sato N, Kamano T, Hayashida Y, Watanabe S. Malignant lymphoma in the ileum diagnosed by double-balloon enteroscopy. *World J Gastroenterol* 2007; 13(24): 3388-3391

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INTRODUCTION

The small intestine, only partially accessible with push-type endoscopy, is the site of most undiagnosed lesions. Enteroscopy with the double-balloon system, an endoscopic technique developed by Yamamoto *et al*[1-3], enables examination of the entire small intestine with simultaneous tissue sampling and a variety of therapeutic interventions. A case is described in which double-balloon enteroscopy was used to diagnose malignant lymphoma in a patient with abdominal pain.

CASE REPORT

A 73-year old man presented with a history of abdominal pain during the previous year. The medical history was otherwise insignificant; physical examination showed a tender point in the right lower abdomen and the vital signs were within normal ranges. Laboratory test results included the following: hematocrit, 33.3% (normal: 40.4%-51.1%); Hb, 10.6 g/dL (14-18 g/dL); serum iron, 20 mcg/dL (80-170 mcg/dL); serum ferritin, 183 ng/mL (30-400 ng/mL); LDH, 133 IU/L (119-211 IU/L); and slL-2R, 1207 U/mL (188-570 U/mL); test of stools for occult blood were positive. Upper endoscopy and colonoscopy with intubation of the terminal ileum were performed, but no abnormality was detected. Computed tomography was performed revealing wall thickening and localized dilation of the ileum in the pelvis (Figure 1). In addition, gallium-67 scintigraphy was performed and collection was detected in the pelvis (Figure 2). A papillary tumor in the dilated ileum was also found using the double-contrast barium study of the small intestine (Figure 3). Enteroscopy with the double-balloon system (EN-450P5/20; Fujinon Co, Ltd, Saitama City, Japan) was performed by anal approach to evaluate the entire intestine.

Full and informed consent was obtained from the patient before the procedure. The patient ingested 2L polyethylene glycol-based solution the day before the enteroscopic examination, which was performed with the patient under anesthesia. A papillary tumor, measuring 50 mm in diameter, was detected at 100 cm proximal to the ileocecal valve (Figure 4). It took 1 h to reach the lesion. Active bleeding was not from the tumor, thus, endoscopic treatment was not required, and no additional lesions were found in the proximal small intestine. The total time for the complete procedure was 2 h. Fluoroscopy was used to position the overtube (total radiography time: 10 min). There were no procedure-related complications. Histological findings from biopsy samples showed large atypical lymphoid lymphocytes. Immunohistochemically,

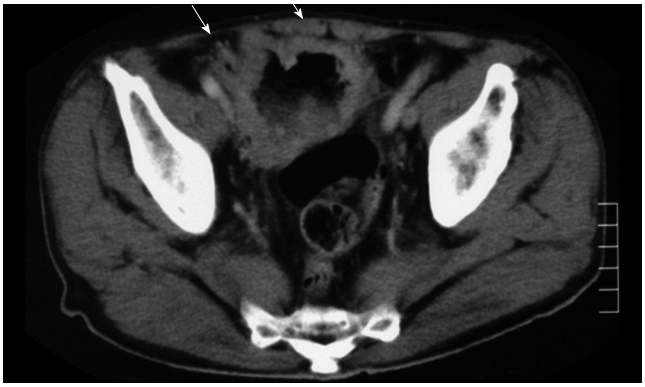

Figure 1 Computed tomography, showing localized dilation of the small intestine (arrows) in the pelvis.

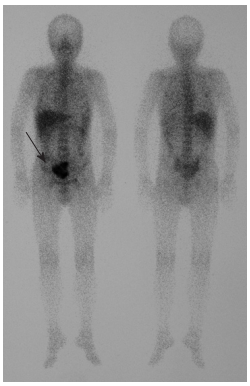

Figure 2 Gallium-67 scintigraphy, showing collection in the pelvis (arrow).

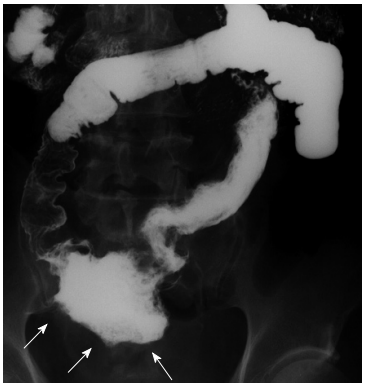

Figure 3 Double-contrast barium study of the small intestine, showing a papillary tumor in the dilated ileum (arrows).

these lymphocytes were positive for CD3 (Figure 5). Therefore, we confirmed the diagnosis of malignant lymphoma, diffuse large B-cell type.

The patient underwent partial resection of the small intestine (180 mm in diameter). Microscopic findings of the resected small intestine showed an elevated lesion (110 mm × 85 mm × 50 mm) with central ulceration (Figure 6). Histologically, the tumor consisted of large atypical lymphoid lymphocytes. The tumor developed from the subserous mucosa and infiltrated bladder. Immunohistochemically, the tumor cells were positive for LCA, L-26, CD3 and UCHL-1. These findings were consistent with the tumor diagnosis of malignant lymphoma, diffuse large B-cell type.

DISCUSSION

Although the gastrointestinal tract is the most common site of extra nodal malignant lymphoma, the small intestine (duodenum, jejunum and ileum) is reported as the site of origin for these lesions in only 15%-30% of gastrointestinal lymphomas[4]. Small-intestinal lymphomas are most often found in the duodenum 11%, jejunum 9%, ileum 29%, and ileocecal area 51%[5]. Histopathologically, B-cell lymphomas and T-cell lymphomas account for 85% and 15% of cases, respectively. Of the subtypes of B-cell lymphoma described in the new World Health Organization (WHO) classification, diffuse large B-cell lymphoma (DLBCL) is the most frequent (68.9%)[6].The overall prognosis of the more advanced stage of primary small intestinal lymphoma is only fair, with an expected 5-year survival of 25% to 30%[7]. In a retrospective analysis of 32 cases of primary small bowel lymphoma treated with either radical surgery plus polychemotherapy (early stages of IE and IIE), the overall 5-year survival was 59%[8].

Malignant lymphoma in the small intestine is difficult to diagnose in the early stages and is often found during emergency surgery in patients with a small bowel obstruction due to two reasons: First, clinical manifestations of small-intestinal lymphoma: anemia, abdominal pain, change in bowel habit, occult blood in stools, GI bleeding, obstruction and perforation (up to 25%) are not specific. Consequently the correct diagnosis is delayed[9]: Second, early diagnosis of malignant lymphoma is also delayed due to the difficulty in accessing the small intestine with conventional upper endoscopy and colonoscopy. Computed tomography (CT) as a frontline tool in the evaluation of abdominal symptoms can identify large masses but is not suitable for the diagnosis of small tumors[10]. Small-intestine radiography for the diagnosis of tumors is often difficult because of the complex looped configuration of the small intestine. CT and small-intestine radiography may be diagnostic, especially in advanced lesions, but they are relatively insensitive for early diagnosis of curable small intestinal tumors. Recently, wireless capsule endoscopy has made it possible to visualize the small intestine[11], but it has limitations such as the lack of air insufflations and the unavailability of rinsing. Consequently, neither of these examinations can be used to obtain biopsy specimens and carry out appropriate treatments.

Double-balloon enteroscopy is a new method of insertion enteroscopy that can explore the small intestine, with alternating steps of inflation and deflation of the two balloons, and alternating insertion of the endoscope and the overtube[2]. This approach enables the operator to visualize the entire small intestine, identify masses at the early stages and take biopsy specimens, the diagnosis of which is an important tool in small intestinal tumor. The exact classification(type and grade) of lymphoma can then be determined and the most suitable treatment can be administered. Consequently, the expected survival rate for patients with malignant lymphoma may increase.

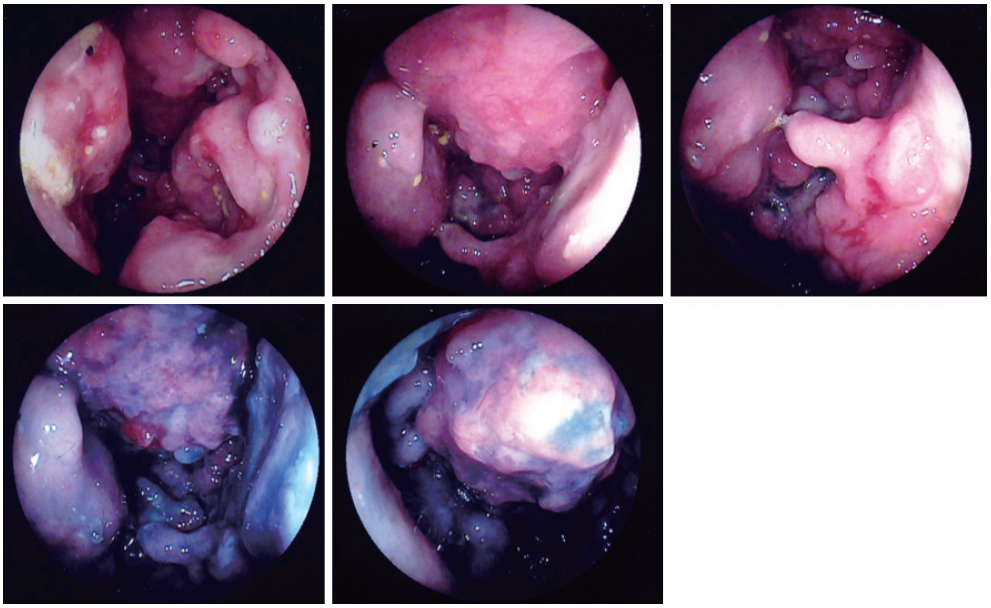

Figure 4 Double-balloon enteroscopic examination, showing a papillary tumor at 100 cm proximal to the ileocecal valve.

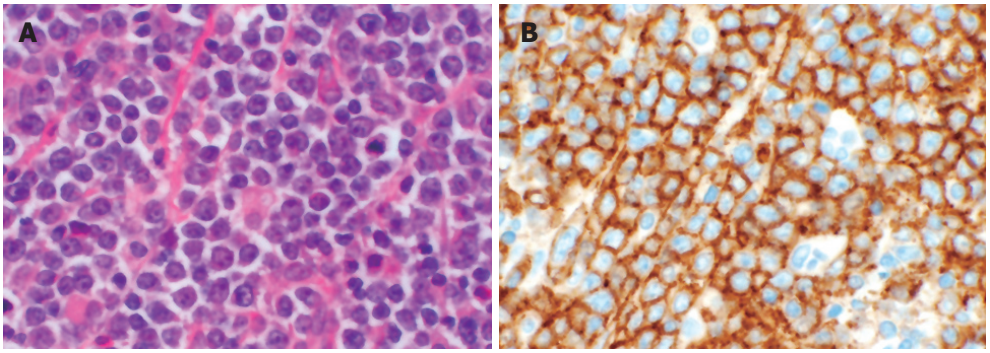

Figure 5 **A**: Histological findings demonstrated large atypical lymphoid lymphocytes, (HE, x 400); **B**: Immunohistochemical analysis showed that these lymphocytes were positive for CD3. (CD3, x 400).

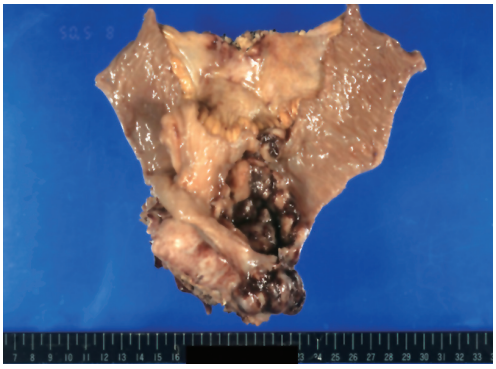

Figure 6 The resected specimen of ileum, showing an elevated lesion (110 mm × 85 mm × 50 mm) with central ulceration.

In conclusion, double-balloon enteroscopy is a very useful method for the diagnosis of small intestinal tumors. Double-balloon enteroscopy represents an important diagnostic and therapeutic tool for small-intestine disease in combination with computed tomography and double-contrast barium study. The present case of anemia and abdominal pain demonstrates its diagnostic value before surgery for obtaining biopsy specimens. Using this method, it may be possible to diagnose small-intestinal lymphoma at the early stages more frequently and administer the most effective treatment based on diagnosis. As a result, the expected survival rate for patients with malignant lymphoma may increase.

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CASE REPORT

Multiple malignant extragastrointestinal stromal tumors of the greater omentum and results of immunohistochemistry and mutation analysis: A case report

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Abstract

To report an extragastrointestinal stromal tumor (EGIST) that occurs outside the gastrointestinal tract and shows unique clinicopathologic and immunohistochemical features. In our case, we experienced multiple soft tissue tumors that originate primarily in the greater omentum, and in immunohistochemical analysis, the tumors showed features that correspond to malignant EGIST. Two large omental masses measured 15 cm x 10 cm and 5 cm × 4 cm sized and several small ovoid fragments were attached to small intestine, mesentery and peritoneum. On histologic findings, the masses were separated from small bowel serosa and had high mitotic count (115/50 HPFs). In the results of immunohistochemical stains, the tumor showed CD117 (c-kit) positive reactivity and high Ki-67 labeling index. On mutation analysis, the c-kit gene mutation was found in the juxtamembrane domain (exon 11) and it was heterozygote. Platelet-derived growth factor receptor (PDGFR) gene mutation was also found in the juxtamememembrane (exon 12) and it was polymorphism. From above findings, we proposed that there may be several mutational pathways to malignant EGIST, so further investigations could be needed to approach this unfavorable disease entity.

Key words: Extragastrointestinal stromal tumor; Greater omentum; c-kit; Platelet-derived growth factor receptor; Mutation

Kim JH, Boo YJ, Jung CW, Park SS, Kim SJ, Mok YJ, Kim SD, Chae YS, Kim CS. Multiple malignant extragastrointestinal stromal tumors of the greater omentum and results of immunohistochemistry and mutation analysis: A case report. *World J Gastroenterol* 2007; 13(24): 3392-3395

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INTRODUCTION

Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal tumors of the gastrointestinal tract and they have unique clinicopathologic features of positive reactivity for CD 117 (c-kit, a transmembrane tyrosine kinase receptor)[1,2]. Mesenchymal tumors of the omentum, mesentery, and retroperitoneum with immunohistochemical features of the GISTs are classified as extragastrointestinal stromal tumors because these organs have no connection with the wall of the gastrointestinal tract[3]. The incidence of EGSTs is less than 10% of the GISTs group[3] and because of their rarity, clinicopathologic and immunohistochemical features of EGSTs are not fully elucidated.

In GIST, several studies have revealed various types of c-kit oncogene mutations, including exon 11 encoding the juxtamembrane domain, exon 9 encoding the extracellular domain, exon 13 and 17 encoding the tyrosine kinase domain[4]. Moreover, the mutations of platelet-derived growth factor receptor (PDGFR) at the juxtamembrane domain (exon 12) and tyrosine kinase domain (exon 18) were reported in some populations of GIST[5]. Also, c-kit and PDGFR mutations in EGIST were reported in some series[6].

Generally, malignant GISTs metastasize to omentum and mesentery, on the other hand, primary tumors may develop in the omentum and mesentery[7,8]. The omental EGIST was very rare and multiple malignant EGISTs from greater omentum had not been reported in recent papers. So, in this paper we describe a case of multiple malignant EGISTs developed in the greater omentum and investigate their immunohistochemical and genetic features.

CASE REPORT

A 64-year old man was admitted to internal medicine department due to right and lower quadrant abdominal

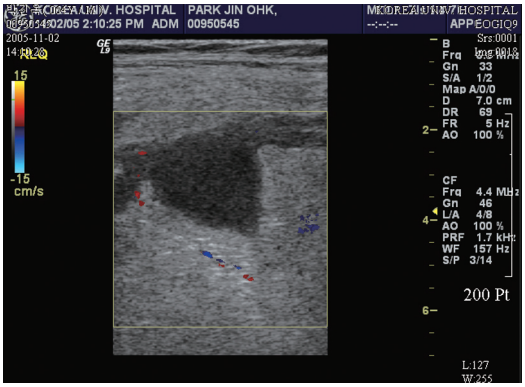

Figure 1 Homo-genous hypovascular hypo-echoic mass lesions in the mesentery on ultrasonography.

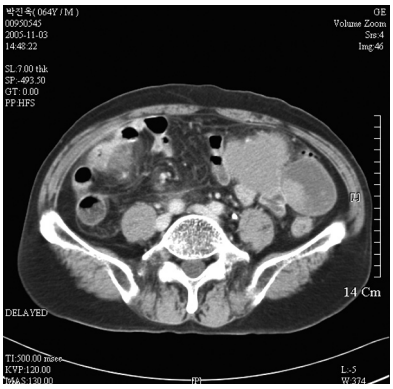

Figure 2 Lesions adjacent to small bowel loop in left abdomen and ill defined low density cystic change on computed tomography.

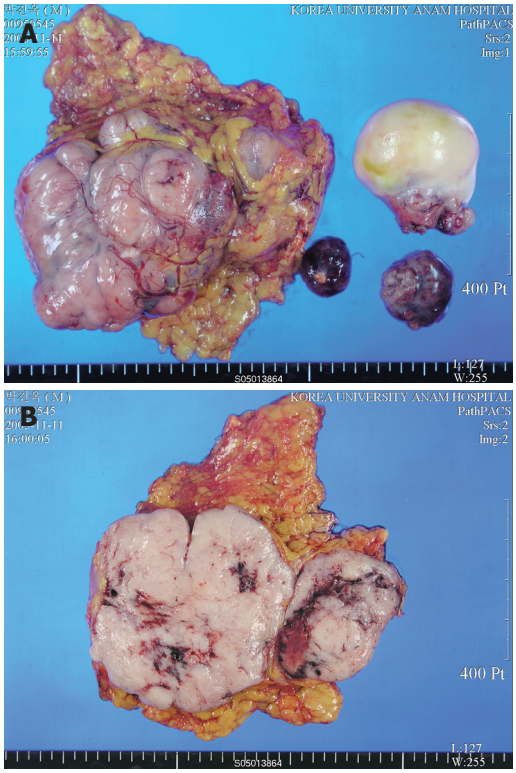

Figure 3 **A**: Gross findings of the specimen: two irregular fragments of omental tissue and several irregular fragments of soft tissue masses; **B**: Diffuse pale brownish flesh-like appearance with multiple hemorrhagic necrosis on cut surface.

mass. Ultrasonography revealed multiple round homogenous hypovascular hypoechoic mass lesions in the mesentery. The masses were variable in size from 1 cm to 2.5 cm in diameter (Figure 1). Preoperative computed tomography revealed 7cm and 5.5 cm sized mass lesions adjacent to small bowel loop in left abdomen and ill defined low density and cystic change in it. There were multiple mesenteric and omental masses in right abdomen that combined ill defined infiltration and streaky density (Figure 2). The patient underwent preoperative ultrasonography-guided biopsy with 18 gauge needle for pathologic diagnosis and the result was highly suspicious for extragastrointestinal stromal tumor and high cellularity, high mitotic count, marked nuclear atypism and necrosis supported the high risk nature of this tumor.

The results of immunohistochemical stains were positive for Vimentin and c-kit (CD117) and negativities for CK (Cytokeratin), SMA (Smooth muscle actin), S-100 protein, Desmin and CD34. The Ki-67 labeling index was increased and the proportion was about 30%-40%.

After transfer to our department, we performed exploration so that multiple tumor de-bulking was performed. On operative findings, multiple tumor masses were resected: about 5 cm sized whitish mass adjacent to small bowel mesentery 80 cm distant distal to Treitz` ligament, two large soft tissue masses of 15 and 5cm sized on greater omentum. A variable sized numerous polypoid masses were noted on the small and large intestines mesentery and peritoneal surfaces; they were removed as much as possible. On 60 cm distant proximal to ileocecal valve, there was conglomerated mass adhescent to small bowel mesentery, so we performed segmental resection of small intestine.

On gross findings, the specimen consisted of two irregular fragments of omental tissue, it measured 15 cm × 10 cm × 5 cm in the larger one and 5 cm × 4 cm × 2 cm in the smaller one and several irregular fragments of soft tissue masses measured 6 cm × 6.5 cm × 4 cm in the largest one (Figure 3A). On section, the cut surface showed diffuse pale brownish flesh-like appearance with multiple hemorrhagic necrosis (Figure 3B).

On immunohistochemical findings, the spindle cells showed cytoplasmic positive reactivity for CD 117 (c-kit) (Figure 4A) and Ki-67 labeling index was increased (Figure 4B). On histologic findings, the tumor was composed of spindle cells showing a less developed fascicular pattern and had a high mitotic activity, that is about 115/50 HPFs mitotic counts (Figure 4C and D).

Mutations of exons 9, 11, 17 of the c-kit gene and those of exons 12 and 18 of the PDGFRA gene were examined, according to the PCR methods. In c-kit mutation, there was heterozygote in exon 11, showing GTT (val.) to GWT (val. + asp.) transition at codon 559 (Figure 5).

The mutation of PDGFRA exon 12 was also found to be a polymorphism, showing CAA (pro.) to CCG (pro.) transition at codon 567 (Figure 6).

After operation, the patient was discharged without any postoperative problem and he was treated with imatinib

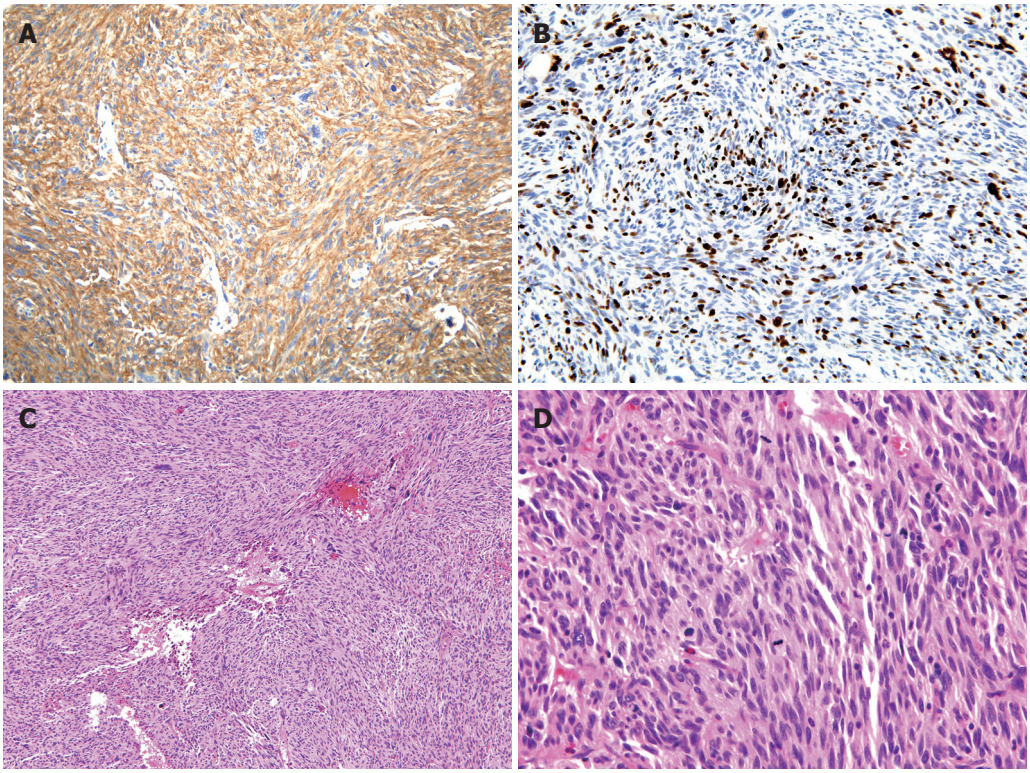

Figure 4 **A**: The spindle cells showed cytoplasmic positive reactivity for CD 117 (c-kit) on immunohistochemical findings; **B**: Ki-67 labeling index was about 30%-40% on immunohistochemical findings; **C**: The tumor was composed of spindle cells showing a less developed fascicular pattern on histologic findings (x 100); **D**: The tumor had a high mitotic activity, about 115/50 HPFs mitotic counts on histologic findings (x 400).

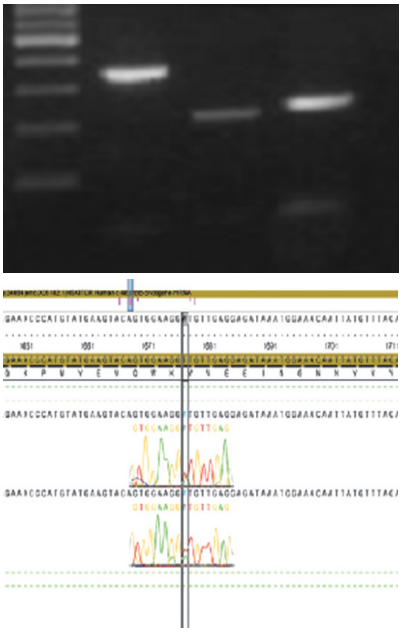

Figure 5 There was a heterozygote on exon 11, showing GTT (val.) to GWT (val. + asp.) transition at codon 559 in c-kit mutation.

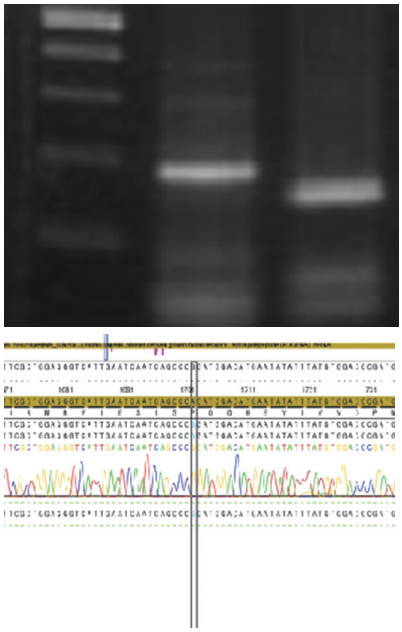

Figure 6 There was a polymorphism on exon 12, showing CAA (pro.) to CCG (pro.) transition at codon 567 in mutation of PDGFRA.

mesylate (Gleevec®, Norvatis Pharma, Basel, Switzerland). There was no recurrence of the tumor at the follow up examination.

DISCUSSION

EGIST is histologically and immunohistochemically similar to their gastrointestinal counterpart but they have an aggressive course resembling small intestinal stromal tumors and they have no or little connection to the abdominal wall or serosal surface of the gastrointestinal tract[3].

Although the histogenesis of EGISTs has not been determined, the expression of the c-kit receptor in E-GISTs suggests either the presence of pacemaker cells outside the gastrointestinal tract or the ability of the mesenchymal elements to recapitulate this phenotype aberrantly. In contrast to studies of GISTs, those of EGISTs did not report any association between the tumor size and outcome. It is believed that this can be best explained by the fact that the majority of EGISTs are large when first detected. This is because small EGISTs are rarely encountered as they hardly ever produce symptoms that lead to detection. Therefore, as Ortiz-Rey *et al* [9]reported, when first detected, many cases of EGISTs can be accessible by a fine needle aspiration biopsy (FNAB).

In our case, we performed FNAB diagnosis and the result was highly suspicious for the diagnosis of

extragastrointestinal stromal tumor. Although GISTs primary in the gastrointestinal tract commonly metastasize in the omentum and mesenteries, often as multiple nodules, GISTs may also occur as primary tumors outside of the gastrointestinal tract proper in other intra-abdominal locations, especially in the omentum and mesentery[10].

In our case, preoperative diagnostic findings were compatible with small intestinal GIST, and small bowel segmental resection was performed because the tumor was adhered to serosal surface of the intestine. But, in pathologic reports, two large omental masses were primary tumors and multiple nodules adjacent to small intestine and peritoneal surface were proved to be metastatic lesions. So, final diagnosis was EGIST.

Prediction of the malignant potential of tumors is important. The size, cellularity, and mitotic activity of EGISTs were reported as the most accurate predictors of adverse outcome[7]. Reith *et al*[3] proposed that in a multivariate analysis mitotic activity and necrosis displayed trends toward independent predictive value.

In our case, the large size (about 15 cm), high mitotic counts (115/50 HPFs) and extensive hemorrhagic necrosis were malignant features, so this tumor belongs to high risk group.

Recently, CD117 (the c-kit protooncogene protein product) has been shown immunohistochemically in EGISTs[4]. CD117 is a transmembrane receptor for a growth factor termed stem cell factor and is expressed in hematopoietic stem cells, mast cells, germ cells, and melanocytes[1]. It is believed to be important in the development and maintenance of these cell populations. Furthermore, CD117 is present in a neural-related population of gastrointestinal mesenchymal cells, the interstitial cells of Cajal located in the muscular layers. These cells are functionally important for gastrointestinal motility and show pacemaker activity[4,6].

Most omental and mesenteric tumors showed histologic features similar to GISTs with elongated spindle cells or epithelioid cells with high cellularity; most of these tumors showed low mitotic activity[1]. Omental and mesenteric GISTs were typically positive for CD117 and less consistently for CD34. They often showed smooth muscle actin reactivity but were virtually negative for desmin and S-100 protein[10].

In our case, the results of immunohistochemical stains were positive for c-kit protein, but showed negative results for CD 34, SMA (smooth muscle actin), and S-100 protein, E-cadherin. In Yamamoto's study[6], in EGIST, high Ki-67 labeling index (≥ 10% HPF) had significantly shorter survival than those with a low index (≤ 10%). Our cases showed high Ki-67 labeling index (about 30%-40%) and that result also showed high-risk nature.

In recent studies, the PDGFRA mutation may play an important role in the small population of EGISTs[6,7,11]. Until now, there was no report that one EGIST tumor showed c-kit and PDGFRA mutations simultaneously. In our case, tumor specimen contained multiple c-Kit and PDGFRA mutations, so it may be the first case reporting two important genetic alterations in one tumor that corresponds to malignant EGIST.

From above findings, we proposed that there may be several mutational pathways to malignant EGIST, so further investigations could be needed to approach this unfavorable disease entity.

STI-571 (Gleevec) is a small molecule that selectively inhibits the tyrosine kinase activity of the abl (bcr-abl), PDGFR, and c-kit [12]. In our case, the patient is now treated with tyrosine kinase inhibitor for multiple and malignant characteristics. Although the effect of STI-571 for PDGFRA-mutant GISTs has not been established, PDGFRA may become a molecular therapeutic target of STI-571 in at least some populations of EGISTs.

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CASE REPORT

A gastrointestinal stromal tumor of the duodenum masquerading as a pancreatic head tumor

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Abstract

Gastrointestinal stromal tumor (GIST) represents the most common kind of mesenchymal tumor that arises from the alimentary tract. GIST is currently defined as a gastrointestinal tract mesenchymal tumor showing CD117 (c-kit protein) positivity at immunohistochemistry. Throughout the whole length of the gastrointestinal tract, GIST arises most commonly from the stomach followed by the small intestine, the colorectum, and the esophagus. Only 3%-5% of GISTs occur in the duodenum, and especially, if GIST arises from the C loop of the duodenum, it can be difficult to differentiate from the pancreas head mass because of its anatomical proximity. Here, we report a case of duodenal GIST, which was assessed as a pancreatic head tumor preoperatively.

Key words: Gastrointestinal stromal tumor; Duodenum; Pancreas head tumor

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INTRODUCTION

Gastrointestinal stromal tumor (GIST) is currently defined as a gastrointestinal tract mesenchymal tumor containing spindle cells (or less commonly epithelioid cells or rarely both) and showing CD117 (c-kit protein) positivity[1-3]. Being a rare entity, GIST represents the most common subset of mesenchymal tumors that arise from the alimentary tract[1]. Primary GIST is solitary rather than multiple. GISTs are common in the stomach (60%-70% of cases), small intestine (30%), and rarely from the the rectum (5%), esophagus, colon and appendix[1-3]. There are also sporadic reports of GIST arising from the omentum, mesentery, and retroperitoneum.

Duodenal GISTs comprise less than 5% of all cases and it can be diagnosed under upper gastrointestinal endoscope due to formation of a gross ulceration in the mucosa or an intramural mass with a centrally ulcerated umblication[4]. However, if there are no specific mucosal changes in the duodenal lumen or presence of anatomical variation, it may be difficult to differentiate a duodenal GIST from a malignant lymphoma, duplication cyst, retroperitoneal tumor, or pancreatic head tumor. We report herein a rare case of a duodenal GIST masquerading as a pancreatic head tumor with history of gastric surgery due to early gastric cancer.

CASE REPORT

An asymptomatic 49-year-old man who underwent gastric resection was admitted to our center for the evaluation of an incidentally found pancreatic head mass. Two years ago, the patient was diagnosed with early gastric cancer and underwent subtotal gastrectomy and gastroduodenostomy. The histological examination revealed 2.5 cm × 1.5 cm sized poorly differentiated adenocarcinoma with confinement to the mucosa, free of resection margin, and free of lymph node involvement. Thereafter, the patient has been examined with endoscopic and radiologic evaluation at regular intervals. On admission, the patient appeared well-nourished, and physical examination findings were unremarkable. Laboratory examinations including peripheral blood cell count, blood chemistry, and serum tumor markers were within normal limits. Duodenal endoscopy, performed by an experienced endoscopist, neither showed mucosal nor submucosal abnormalities in the duodenum (Figure 1), nor any evidence of gastric

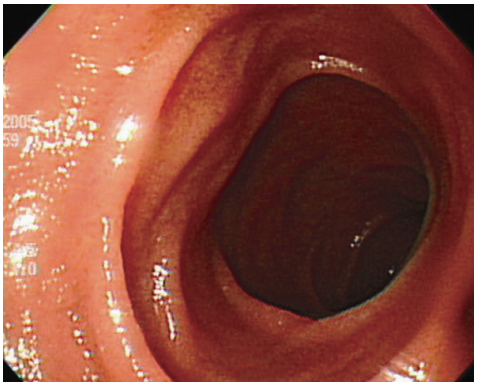

Figure 1 Duodenal endoscopy neither showed an intraluminal mass nor a mucosal abnormality under good visualization to the fourth part of the duodenum.

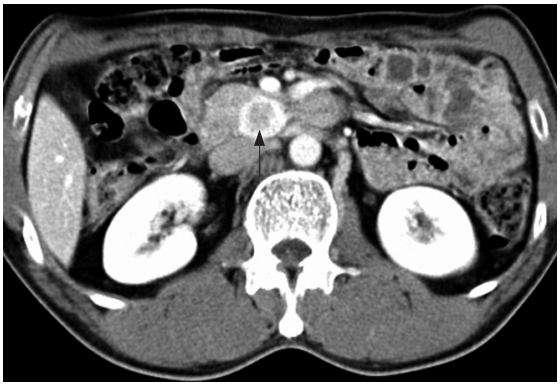

Figure 3 The CT scan of upper abdomen showed an intense homogenously enhancing tumor with 2.3 cm in diameter in the pancreatic head region (arrow).

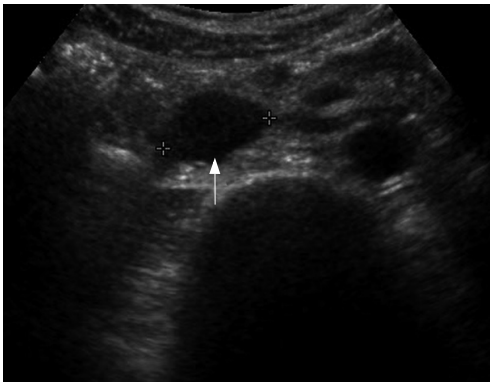

Figure 2 Abdominal ultrasound showed 2.3 cm sized, oval-shaped hypoechoic solid nodule around the uncinate process of the pancreas (arrow).

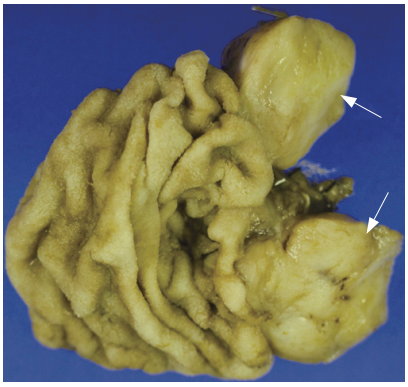

Figure 4 Macroscopic appearance of the resected specimens; a round yellow gray colored tumor of cut surface (arrows) indicated the submucosal mass growing outwards towards the duodenal lumen.

cancer recurrence in the remnant stomach. Abdominal ultrasonography revealed 2.3 cm sized, oval-shaped hypoechoic solid nodule around the uncinate process of the pancreas (Figure 2) and the computed tomography scan showed a tumor in the uncinate portion of the pancreas, measuring 2.3 cm in diameter. The tumor was well-demarcated and strongly enhanced on contrast-enhanced CT images (Figure 3). On the basis of above findings, the presence of a tumor in the uncinate process of the pancreas, most likely a non-functioning islet cell tumor, was strongly suspected.

At laparotomy, a 2.3 cm sized soft tissue lesion was found lying between the second and third parts of the duodenum and mobilization of the duodenum revealed a tumor protruding from the duodenal wall, posterior to the head of the pancreas with a minimal adhesion. After dissection of the inferior portion of the pancreas head from the duodenal wall, segmental resection of the duodenum and duodenojejunostomy was performed.

Macroscopic examination of the surgical specimen showed a well-demarcated round yellowish mass measuring 2.3 cm in maximum diameter (Figure 4). The tumor arose from the muscularis propria of the duodenum, and extended outwards to the lumen. Under microscopic examination, the tumor showed spindle cells with rare mitotic figures on the H&E stained tissue sections (Figure 5). Immunohistochemical study revealed positive staining for c-kit protein and CD34, but negative reactions to α-smooth muscle actin (Figure 6). Based on the above findings, the tumor was finally diagnosed as a GIST with low-grade malignancy originating from the duodenum.

The postoperative course proved unremarkable and further treatment was felt to be unnecessary. Clinical follow-up was on-going at the time of this report without any evidence of recurrence.

DISCUSSION

Gastrointestinal stromal tumors are low-grade malignant mesenchymal tumors of gastrointestinal tract and are believed to originate from the neoplastic transformation of the interstitial cells of Cajal from their precursors[5]. Mesenchymal tumors developing from the gastrointestinal tract had been believed to be of neuronal or muscle cell origin. However, recent immunohistochemical studies have shown that most tumors showing no typical staining for muscle type or neuronal type cells could be classified as GISTs, which are now defined as tumors showing the expression of a specific, c-kit proto-oncogene product (CD117); that is, a receptor tyrosine kinase protein (KIT)[6,7]. Approximately 70% of all GISTs are positive for the CD34 protein, which is a hematopoietic progenitor cell antigen[8], while 20%-30% are positive for SMA, 10% are

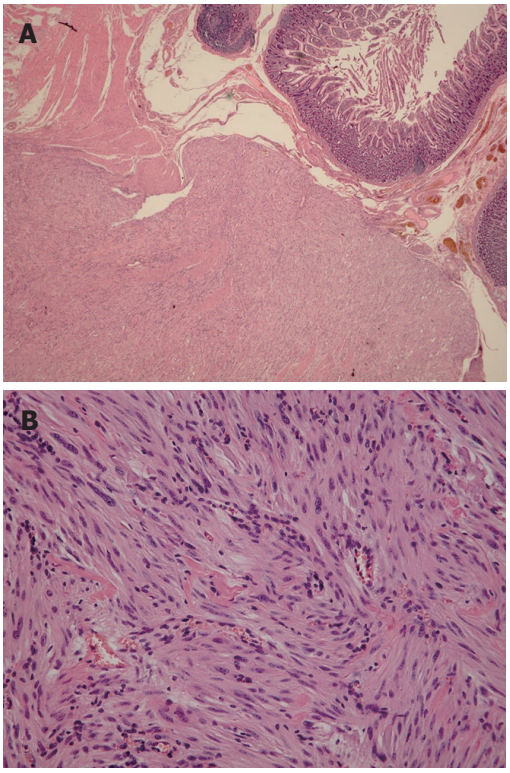

Figure 5 A well-defined subserosal duodenal GIST showing interlacing fascicles of the spindle cells with elongated cytoplasm (H&E stain; **A**: × 20, **B**: × 200).

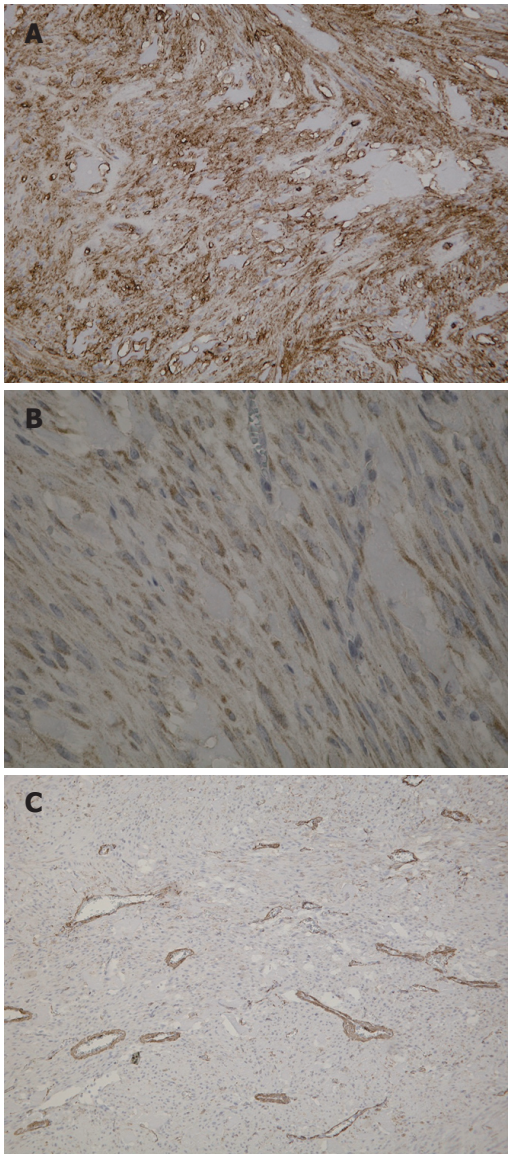

Figure 6 The tumor cells react positively for CD34 (**A**: × 200) and c-kit (**B**: × 200) while negative for SMA (**C**: × 100) on immunohistochemistry.

positive for S-100 protein, and fewer than 5% are positive for desmin, which is the intermediate filament protein of smooth, skeletal, and cardiac muscle cells[1].

GISTs can occur throughout the gastrointestinal tract, but may also occur in the peritoneum and extragastrointestinal sites. Duodenal GISTs most frequently involve the second portion of the duodenum, followed by the third portion, fourth portion, and first portion[4]. Although many of duodenal GISTs extend from submucosal or muscularis propria to external aspects (58 tumors out of 156 duodenal GISTs), most of them comprise a gross ulceration of the mucosa with a component that bulged underneath the mucosa coincidentally, which helps in the detection of the tumor upon endoscopic examination[4]. With the present patient, the tumor showed transmural growth outwards towards the lumen, and there was no specific intraluminal mucosal changes or visible mass in the duodenum.

An extensive review of the English-language literature regarding GISTs revealed only one report of a duodenal GIST with the unusual features observed in this patient. The preceding report showed minimal epithelial changes in the intraluminal surface of the duodenum with the tumor exhibiting transmural growth into the pancreatic tissue[9]. These features are rare, and it was difficult to differentiate the organ from which the tumor originated, on the US and CT images. Thus, at first, we considered the tumor to be a nonfunctioning islet cell tumor originating in the pancreatic head indicated by its well enhanced characteristics of the tumor on contrast-enhanced CT images.

The clinical presentations of duodenal GIST are highly variable according to their size and the existence of mucosal ulceration. According to the previous report, duodenal GISTs most commonly present GI bleeding, epigastric pain, palpable mass, and intestinal obstruction[4]. Small tumors, especially which are not accompanied by mucosal ulceration, are usually incidental findings upon operation, endoscopy or imaging studies for other reasons. Endoscopy may be diagnostic in duodenal GIST. However, in this case, it was unable to detect the duodenal tumor despite adequate visualization of the duodenum. This may have been due to the relatively smaller diameter and outward growth of the tumor without forming intraluminal mass or centrally ulcerated umbilication.

CT and MRI seemed to be the best imaging modalities for assessment of the primary lesion and detection of metastases. Although their relative usefulness depends on the site of the GIST, CT is particularly useful for small bowel or omental GISTs that are not accessible by endoscopy[10]. On CT, GISTs may vary from small homogenous masses to large necrotic masses. Small tumors typically appear as sharply-marginated, smooth-

walled, homogenous, soft tissue masses with moderate contrast enhancement. On the contrary, large tumors tend to have central necrosis and cavitations, as well as heterogenous enhancement[11]. Lymphadenopathy is unusual with GISTs and, if present, should raise an alternative diagnosis of lymphoma or adenocarcinoma[10]. In the present patient, although the tumor had consistent imaging features of GIST, it did not show a typical growth pattern of duodenal GISTs and the tumor was located at the C loop of the duodenum that abuts the head and uncinate process of the pancreas. With such features, it is difficult to differentiate GISTs from other types of pancreatic masses, especially pancreatic islet cell tumors, which also show a hypervascular mass on CT scan.

Optimal surgical treatment of GISTs entails complete removal of the tumor with clear surgical margins including adjacent involved organs[12,13]. Because local and regional lymph node involvement are infrequent in GISTs, smaller gastric GISTs are adequately treated utilizing wedge resection instead of formal gastrectomy[12]. Similarly, local or segmental resection should be considered in the treatment of small duodenal GISTs when technically feasible[13]. Resection of the tumor with primary closure can be performed for smaller lesions if the resulting lumen is adequate and the ampulla of Vater can be preserved. Segmental duodenectomy with duodenojejunostomy can be performed for larger tumors located in the infra-ampullary portion. When the duodenal GISTs are located at the second portion of the duodenum, major resection *via* a pancreaticoduodenectomy or a pancreas-sparing duodenectomy is indicated. In this patient, we performed segmental resection of the duodenum with duodenojejunostomy because the tumor was located below the second portion of the duodenum and was relatively larger in size (> 2 cm) as a duodenal GIST.

Based on Fletcher's criteria defining the risk of malignancy[2], we assessed the degree of malignancy of our patient as a low grade malignancy because the tumor was smaller than 5 cm and had rare mitotic features. The location of the tumor, however, may be one of the important prognostic factors, especially in GISTs arising in the small intestine, which showed more malignant behavior even at low level mitotic activity than GISTs developing in the stomach[14]. Additionally, Miettinen *et al*[4] reported that duodenal GISTs with > 2 cm but not > 5 cm in diameter and a low mitotic rate had a low frequency of malignant behavior even ten years after excision of the primary tumor. Thus, the occurrence of relapse necessitates long-term follow-up for this patient and it is hoped that a consensus on duodenal GIST prognosis and predictive markers will be established in the near future.

Our patient also had an early gastric cancer and resulting in a subtotal gastrectomy. Thereafter, a duodenal GIST was discovered incidentally. Some reports have described the concurrence of GISTs and other malignant tumors such as renal cell carcinoma, gallbladder cancer, gastric cancer, and gastric lymphoma[15]. Furthermore, about 10% of patients with GISTs have a history of synchronous or metachronous malignant neoplasms. However, the relation of GISTs with another tumor is still unknown as to whether this phenomenon is caused by genetic abnormalities or random coincidences in patients with GISTs. Supplemental genetic studies of the relation between GISTs and other malignant neoplasms should be performed.

In summary, we have described a rare duodenal GIST with an extraluminal growth mimicking a pancreatic head tumor developed two years after the resection of early gastric cancer.

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