Furthermore, the finding that presacral recurrences develop despite presacral intraoperative radiotherapy[5] makes postoperative migration of tumour cells to the presacral subsite more plausible. It is also possible that tumour spillage from positive margins and leakage from lateral lymph flow routes might occur simultaneously.

The purpose of the present study was to obtain detailed anatomical knowledge about the lateral lymph nodes, in order to determine whether these might play a role in the development of local recurrence. This was achieved by studying serial sections of human fetuses and making a three-dimensional reconstruction to enable analysis of the topographical relationship between the lateral lymph nodes and the mesorectal package. Investigation of serially sectioned fetal pelves has several advantages over conventional cadaver dissections. Blood and lymph vessels and nerves are relatively large in fetuses and therefore easier to identify. In addition, pelvic connective and fatty tissues are less prominent than in adults. Finally, the topographic relationships of the different tissues remain undisturbed in histological sections.

Methods

Ten serially sectioned human fetal pelvises from collections in the Department of Anatomy and Embryology of Leiden University Medical Centre and the Academic Medical Centre in Amsterdam were studied. Fetuses were obtained with informed consent after legal abortion or miscarriage. There were six female and four male fetuses, with ages ranging from 10 to 16 weeks. The transverse sections (10 μm) were stained with haematoxylin and azophloxin, haematoxylin and eosin, or azar. The relationships between the mesorectum and surrounding vessels, autonomic nervous system and lymph vessels were studied at high magnification in all ten fetuses. One fetal pelvis was additionally stained immunohistochemically for lymph tissue (lymphatic vessel endothelial receptor (LYVE) 1 antibody, 1:50 dilution). Serial images of sections were recorded with a digital camera and acquired at a constant distance. Lymph tissue, blood vessels, neuronal tissue and other structures were marked individually in these images. Three-dimensional reconstructions of the fetal pelvis were prepared with the use of the Amira® software package version 4.0 (Template Graphics Software; Visage Imaging, San Diego, California, USA)[9–11].

Results

In human fetuses at the stages used in this investigation the mesorectum could be recognized by the compactness of the connective tissue around the rectum (*Fig. 1*). Overall, the lateral lymph tissue was prominent and located bilaterally to the rectum, surrounding the internal and external iliac arteries. There was no lymph node tissue in the presacral area. The parietal and visceral fascias were connected at the anterior and posterior side of the rectum. On the lateral sides there was no clear compact tissue, but loose mesenchyme-containing vessels, nervous tissue and lymph node tissue.

At high magnification the relationship between the mesorectum and middle rectal vessels could be identified clearly. The middle rectal artery entered the mesorectum at the level below the peritoneal reflection in all ten fetuses. The arteries were always accompanied by middle rectal veins, and passed the mesorectum on the anterior side (*Fig. 1a–d*). In nine of the ten fetuses, the middle rectal vessels could only be found unilaterally. At the level where the middle rectal artery and vein passed the mesorectum, these vessels were accompanied by lymph vessels (*Fig. 1e*). In the proximity of the mesorectum, the autonomic nervous system and the lymph vessels had a very close relationship. The medial part of the inferior hypogastric plexus that innervated the rectal wall was surrounded by lymph tissue (*Fig. 1f–i*). In all ten fetuses lymph vessels entering the mesorectum were noted (*Fig. 1j*). There were no connections between the mesorectal lymph node system and the urogenital region. There were no differences between the male and female lymph node systems.

The topographical relationship between the rectum, autonomic nerve plexus and lateral lymph tissue is visualized in a three-dimensional reconstruction in *Fig. 2*. The rectum, enclosed by the mesorectum, is divided into upper and lower parts by the peritoneal reflection. Directly anterolateral to the mesorectum the inferior hypogastric plexus is located on both sides. The lateral lymph node basins, surrounding the internal and external iliac vessels, are located laterally to the autonomic nerve plexus. Only at the level below the peritoneal reflection do the lymph vessels, middle rectal vessels and nerve strands conjoin (often referred to as the 'lateral ligament'), and pass to the mesorectum.

Discussion

Since the introduction of TME in rectal cancer surgery local recurrence rates have been reduced to 5–15 per cent; 30–56 per cent of these recurrences are located in the presacral space, independent of treatment modality[2–5]. Known risk factors for local recurrence are low tumour location, absence of neoadjuvant therapy, APR surgery, higher tumour node metastasis (TNM) stage, involvement

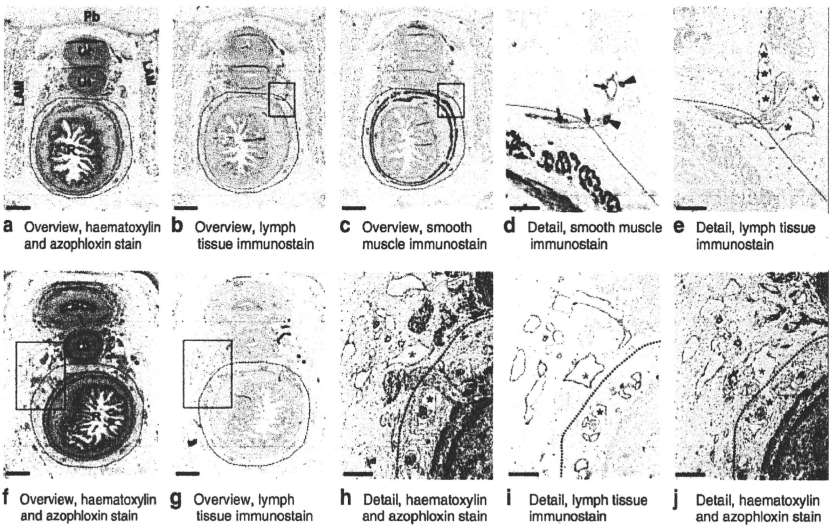

Fig. 1 (Immuno)histochemically stained serial sections of a female fetus (12 weeks of gestation). Sections were cut in the transverse plane. All images are shown with ventral side up. The dotted lines around the rectum illustrate the border of the mesorectum. In fetuses at this stage the mesorectum is not contained within a clear fascia. The mesorectum can be recognized because of the more compact connective tissue in the mesorectum than outside (images **f** and **h** most clearly show the difference between the loose connective tissue outside the mesorectum and the more compact connective tissue of the mesorectum). **a–e** Sections taken at the level of the middle rectal artery/vein. **a** Overview image of the pelvic organs and surrounding tissue stained with haematoxylin and azophloxin. Pb, pubic bone; Ur, urethra; Ut, uterus; LAM, levator ani muscle; R, rectum. **b** Consecutive section stained immunohistochemically for lymph tissue (lymphatic vessel endothelial receptor (LYVE) 1 antibody, 1 : 50 dilution). **c** Section consecutive to that in **a** and **b** stained immunohistochemically for smooth muscle tissue (α-smooth muscle actin antibody, 1 : 1500 dilution). Note the staining of smooth muscle in the rectal wall. **d** Detail of panel **c** illustrating the middle rectal artery (arrowheads) and middle rectal vein (arrows). The blood vessels can be identified by their positive staining for α-smooth muscle actin. Note that the middle rectal vein courses through the border of the mesorectum into the mesorectal space. **e** Detail of panel **b** (section consecutive to that of **c**/**d**) illustrating lymph tissue (asterisks) surrounding the middle rectal artery and vein after staining with LYVE-1 antibody. Note that the lymph tissue also enters the mesorectal space, together with the middle rectal artery/vein. **f–j** Sections taken at the level where the autonomic nerves approach the rectum. **f** Overview image of the pelvic organs and surrounding tissue stained with haematoxylin and azophloxin. Bl, urinary bladder. **g** Consecutive section stained immunohistochemically for lymph tissue. **h** Detail of panel **f** illustrating an autonomic nerve (between the grey and black asterisks, which indicate lymph vessels) that innervates the rectal wall. Note that the autonomic nerve courses through the border of the mesorectum into the mesorectal space. **i** Detail of panel **g** illustrating the lymph tissue surrounding the autonomic nerve that innervates the rectum. Note the two marked lymph vessels (asterisks). The lymph vessel marked with a black asterisk is situated inside the mesorectum, and that marked with a grey asterisk is outside the mesorectum. **j** Haematoxylin and azophloxin-stained section consecutive to that in panel **i**. At this level the lymph vessels (grey and black asterisks) connect through the mesorectal border. Scale bars: 0·5 mm in **a**, **b**, **c**, **f** and **g**; 0·2 mm in **h**, **i** and **j**; 0·1 mm in **d** and **e**

of the circumferential resection margin and, in some studies, anastomotic leakage[12,13].

The purpose of the present study was to obtain anatomical knowledge about the lateral lymph nodes and their connection to the mesorectal lymph tissue, in order to gain insight into the mechanism of development of local recurrences, irrespective of the known risk factors. Presacral local recurrence results in especially disastrous

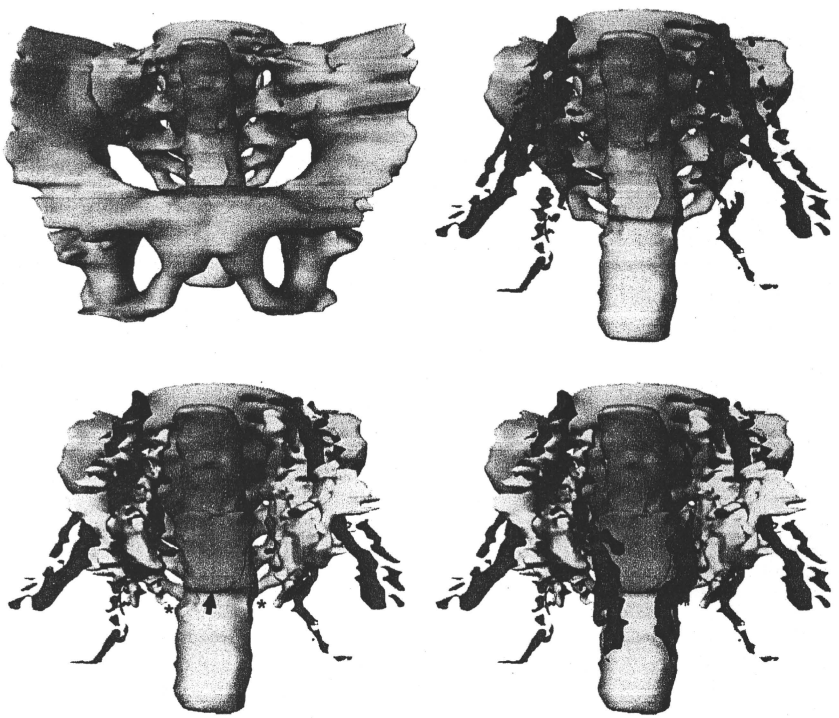

Fig. 2 Three-dimensional reconstruction of a female fetal pelvis (12 weeks of gestation) illustrating the topographical relationship between the lateral lymph tissue and the rectum. The level of the peritoneal reflection is marked with an arrow. The level at which the lateral lymphatics are connected to the mesorectal lymphatics below the peritoneal reflection is marked by asterisks. This is also the level of the sections shown in *Fig. 1* (level of the 'lateral ligament'). Colour codes: grey, pelvic bone and sacrum; light blue, rectum surrounded by mesorectum; orange, peritoneum; green, autonomic nerve plexus; red, arteries; dark blue, veins; yellow, lateral lymphatic tissue

outcomes[14] and understanding how it develops may lead to preventive strategies.

In serial sections from ten human fetuses and in the three-dimensional reconstruction it was demonstrated that the lateral lymph node tissue comprises a major proportion of the pelvic tissue volume. Connections between the mesorectal and (lateral) extramesorectal lymph node system exist, located below the peritoneal reflection on the anterolateral side of the fetal rectum. At this site middle rectal vessels pass to and from the mesorectum, and the branches of the autonomic nervous system bridge to innervate the rectal wall. The lymph connection along the middle rectal vessels has been demonstrated previously by ink injection into the lymphatics of stillborn infants[15].

During TME, after posterior and anterior dissection between the visceral and parietal fascia, the lateral phase is the most important in sparing the autonomic nervous system. At the lateral edges of Denonvilliers' fascia the

inferior hypogastric plexus divides into branches to the rectum (lateral ligament) and the genitourinary organs. By pushing the rectum contralaterally and dorsally, and with sharp dissection along the medial side of the lateral ligament, only the rectal branches of the nerves are dissected. It should be noted that the 'lateral ligament' is not a ligament in the anatomical sense, but in the surgical sense[16]. It is a condensation of loose connective tissue around the blood vessels (middle rectal artery and veins), autonomic nerves and lymph vessels that approach the mesorectum.

The authors hypothesized that, when mobilizing the rectum during surgical excision, lymph fluid and tumour cells flow into the lateral lymph node system. As this lateral lymph tissue is left behind in a standard TME and partly damaged during sharp dissection of the lateral ligament, one would expect the basins to start leaking after the procedure. This lymph fluid, collected presacrally in a seroma, might give rise to local tumour recurrence.

Although the present findings provide a reasonable anatomical explanation, they cannot prove this hypothesis. A possible way of proving this theory would be to isolate tumour cells from lymph fluid, for example collected from a presacral drain, after radical resection of rectal cancer. Efforts are currently being made at Catharina Hospital to conduct such tests, but they are challenging owing to the complicated techniques used to demonstrate the presence of tumour cells in fluids.

If it is true that lateral lymph node metastases play a major role in the development of local recurrence, the question of appropriate therapeutic options remains. In the West preoperative (chemo)radiotherapy combined with TME is the standard treatment. It is unclear whether radiotherapy can sterilize extramesorectal tumour particles. Radiotherapy can possibly reduce local recurrence rates in the lateral pelvic subsite[6]. However, even after radiotherapy presacral local recurrences are still common[6,17].

In the East, initiated mainly by surgeons in Japan, lateral lymph node dissection (LLND) is the standard operation for locally advanced rectal cancer[18,19], without the use of (neo)adjuvant treatment. In this technique, all lymph nodes along the internal and external iliac and obturator arteries are resected, with lateral lymph node positivity in up to 20 per cent of patients[20,21]. A bilateral lymph node dissection possibly prevents more local recurrences than a unilateral dissection, not only in the lateral area but also at the presacral subsite[8]. However, in obese Western patients, a LLND is more difficult and can lead to excess morbidity.

It seems that the lateral lymph nodes are neglected in rectal cancer therapy in the West. Although widespread application of LLND is probably not an option, reducing the radiation volume to the lateral basins with intensity-modulated radiation therapy might give rise to problems if positive lateral lymph nodes are not suspected and thus not irradiated. Furthermore, with current magnetic resonance imaging, clearly involved or suspected lateral lymph nodes are sometimes identified. The risk of disseminated disease is high and the prognosis unfavourable for those with positive lateral lymph nodes. For these patients it may be wise to consider a combination of neoadjuvant chemoradiotherapy, LLND and systemic therapy.

Acknowledgements

The authors declare no conflict of interest.

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Commentary

Origin of presacral local recurrence after rectal cancer treatment (*Br J Surg* 2010; 97: 1582–1587)

This article underlines the importance of the lateral lymphatics in the presence of rectal cancer. It is a further publication in the recent literature highlighting that Western surgeons elect to leave these lymphatics, partly because of the additional morbidity of a wider dissection in the 'Western pelvis'. This is contrary to a philosophy commonly adhered to by Japanese surgeons that an extended pelvic lymphadenectomy reduces local recurrence. This is undoubtedly a controversial area, and the current focus remains on the quality of surgery being performed, and the role of radiotherapy with or without chemotherapy. Efforts in these areas have led to a significant reduction in local recurrence rates, in spite of any role the lateral lymphatics may play in the development of pelvic recurrence.

Numerous publications have identified the presence of lateral pelvic lymphatics, and demonstrated the drainage relationship with the mesorectal lymphatics. These include the original Prussian blue dye injection studies by Gerota in 1895[1] and Villemin and colleagues in 1925[2], through to rectal lymphoscintigraphy studies by Kaplan[3], Bucci and colleagues[4] and Miscusi *et al.*[5] in the 1980s. There have also been two publications relating to the pelvic lymphatic anatomy in early development. In 1927 Senba[6] reported on his study of 200 fetuses, and in 1950 Blair and colleagues[7] published results in infant cadaver dissections, although this paper is more known for showing that rectal lymphatics did not cross the dentate line.

Postoperative serum α-fetoprotein level is a useful predictor of recurrence after hepatectomy for hepatocellular carcinoma

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Received December 18, 2009; Accepted March 3, 2010

DOI: 10.3892/or_00000888

Abstract. We evaluated the clinical value of perioperative α-fetoprotein (AFP) and des-γ-carboxy prothrombin (DCP) levels in predicting recurrence of hepatocellular carcinoma (HCC) after curative resection, with a focus on the time course as surveillance tools. A total of 165 consecutive HCC patients who had undergone curative hepatectomy at our institution from 2005 to 2007 and whose serum AFP and DCP had been measured before and after hepatectomy were included in this study. The minimum postoperative levels within a 4-month period were used for analysis. Among the patients with a positive level of AFP before operation, the number of patients whose AFP level did not change from positive to negative after operation in the group with recurrence exceeded that in the group without recurrence (48/60, 80.0% vs. 4/23, 17.4%), and the difference was significant (P<0.001). Minimum postoperative AFP level was found to be a significant independent risk factor for recurrence by multivariate analysis (P<0.001). There was no statistically significant correlation between AFP level and grade of hepatitis activity (P=0.599). Postoperative AFP level is a useful tool for predicting recurrence after curative hepatectomy. A positive level of AFP after operation might suggest a site of residual viable cancer. The need for effective adjuvant therapy and close follow-up is suggested in patients with a positive postoperative AFP level.

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Abbreviations: AFP, α-fetoprotein; DCP, des-γ-carboxy prothrombin; HCC, hepatocellular carcinoma; CT, computed tomography; MRI, magnetic resonance imaging; US, ultrasonography; TMN, tumor-node-metastasis; UICC, International Union against Cancer; PPV, positive predictive value; NPV, negative predictive value; ALT, alanine aminotransferase; AFP-L3, *Lens culinaris* agglutinin-reactive α-fetoprotein; GPC3, glypican-3; HBsAg, hepatitis B s antigen; anti-HCV, anti hepatitis C virus antibody; AST, aspartate aminotransferase; T. Bil, total bilirubin; Alb, albumin; ChE, cholinesterase; PLT, platelet; PT, prothrombin time; ICG-R15, indocyanine green-retention at 15 min

Key words: hepatocellular carcinoma recurrence, α-fetoprotein, Des-γ-carboxy prothrombin, tumor marker, hepatectomy

Introduction

Primary liver cancer, which consists predominantly of hepatocellular carcinoma (HCC), is the fifth most common cancer worldwide and the third most common cause of cancer mortality, and is becoming more prevalent not only in South-East Asia and Africa but also in Western countries (1-4). Therefore, great interest in HCC has recently developed all over the world.

Surgical resection is the most effective treatment for curable HCC. Although the short-term prognosis of HCC patients has improved due to advances in surgical technique and perioperative management, the long-term prognosis remains far from satisfactory due to frequent recurrence because of not only metastasis from the primary tumor but also multicentric carcinogenesis based on underlying hepatitis or cirrhosis. Therefore, control of recurrent disease is a major challenge in HCC treatment and, especially, the establishment of effective adjuvant therapy to prevent recurrence is required. However, there is no universal consensus at present (5). Secondly, because the outcome in cases of HCC recurrence is likely to be improved by early detection, postoperative surveillance is also required.

Serum levels of α-fetoprotein (AFP) and des-γ-carboxy prothrombin (DCP) are well known as tumor markers of HCC (6). Although many study groups have indicated that preoperative levels of these tumor markers were prognostic factors for HCC (7-11), the clinical usefulness of postoperative levels for early detection of HCC recurrence remains unclear.

This study evaluated the clinical value of perioperative serum AFP and DCP levels in predicting HCC recurrence after curative resection, with a focus on the time course as surveillance tools.

Materials and methods

Patient selection. From January 2005 to December 2007, 192 consecutive patients with HCC underwent hepatectomy at the

Table I. Baseline characteristics of study patients (n=165).

Age (years)	67.2±8.6
Gender (male/female)	138 (83.6)/27 (16.4)
Viral infection [HBsAg(+)/anti-HCV(+)/both(+)/both(-)]	30 (18.2)/97 (58.8)/6 (3.6)/32 (19.4)
Diabetes (present/absent)	44 (26.7)/121 (73.3)
Tumor occurrence (initial/recurrent)	108 (65.5)/57 (34.5)
Child-Pugh classification (Class A/B/C)	150 (90.9)/15 (9.1)/0 (0.0)
AST (IU/l)	56.2±38.1
ALT (IU/l)	57.2±44.6
T. Bil (mg/dl)	0.9±0.3
Alb (g/dl)	3.9±0.3
ChE (IU/l)	247.0±71.0
PLT ($x10^3/mm^3$)	15.9±16.9
PT (%)	78.7±10.9
ICG-R15 (%)	17.2±10.4
Preoperative AFP (ng/ml)	1464.0±6038.2
Minimum postoperative AFP (ng/ml)	695.7±7755.1
Preoperative DCP (mAU/ml)	1539.8±6204.2
Minimum postoperative DCP (mAU/ml)	39.9±125.3
Tumor size (cm)	3.9±2.9
No. of nodules (solitary/multiple)	108 (65.5)/57 (34.5)
Histological differentiation (well/moderate/poor)	29 (17.6)/92 (55.8)/44 (26.7)
Macroscopic vascular invasion (present/absent)	7 (4.2)/158 (95.8)
Microscopic vascular invasion (present/absent)	43 (26.1)/122 (73.9)
Intrahepatic metastasis (present/absent)	14 (8.5)/151 (91.5)
Stage (UICC) (I/II/III/IV)	86 (52.1)/62 (37.6)/15 (9.1)/2 (1.2)
Operative procedure	
(trisegmentectomy/central bisegmentectomy/	2 (1.2)/2 (1.2)/
hemihepatectomy/segmentectomy/	18 (10.9)/15 (9.1)/
subsegmentectomy/partial resection)	14 (8.5)/114 (69.1)
Surgical margin (mm)	2.7±4.2
Histological findings of non-cancerous lesions (liver cirrhosis/	60 (36.4)/90 (54.5)/15 (9.1)
chronic hepatitis/normal liver)	

Continuous variables are expressed as mean and standard deviation. Values in parentheses are percentages.

National Cancer Center Hospital East, Japan. Among them, 165 patients were enrolled in this study, excluding 14 who underwent non-curative resection, 9 who were lost to serial follow-up, and 4 who took oral warfarin, a DCP-inducing agent. Baseline characteristics of the patients are shown in Table I. None of the patients in this study received post-operative adjuvant therapy, including interferon.

Diagnosis of HCC. HCC was diagnosed using dynamic computed tomography (CT) or magnetic resonance imaging (MRI), considering hyper-attenuation in the arterial phase with washout in the late phase to indicate definite HCC (12). Intraoperatively, ultrasonography (US) was performed to determine whether other nodules were present in the liver or not (13). All nodules were confirmed to be HCC histopathologically after surgical resection. Pathological stage was assigned according to the tumor-node-metastasis (TNM) classification of the International Union against Cancer (UICC) (14), and curative resection was defined as a negative surgical margin histopathologically. Tumor recurrence was defined as a newly developed lesion on CT or MRI.

Measurement of serum AFP and DCP concentrations. Serum AFP and DCP concentrations were determined within 1 month before operation and at least once within a 4-month period after operation, using a commercially available electrochemiluminescence immunoassay kit (Roche Co., Tokyo, Japan) and chemiluminescent enzyme immunoassay kit (Eisai Co., Tokyo, Japan), respectively. The minimum postoperative level was used for analysis, and the levels of the tumor markers before and after operation were compared. In this study, the cut-off levels for AFP and DCP were set as 10 ng/ml and 40 mAU/ml, respectively.

Statistical analysis. Continuous variables were expressed as mean and standard deviation. The number of patients whose

Table II. Sensitivity of α-fetoprotein (AFP), des-γ-carboxy prothrombin (DCP) and combination of both.

	AFP (%) (Cut-off level; 10 ng/ml)		DCP (%) (Cut-off level; 40 mAU/ml)		Combination of AFP and DCP (%)	
Sensitivity before operation	83/165	50.3	101/165	61.2	130/165	78.8
Sensitivity at recurrence	63/114	55.3	46/114	40.4	82/114	71.9

tumor marker levels changed from positive to negative after operation was compared using χ^2 test. Recurrence rates were analyzed according to the Kaplan-Meier estimate, and differences between subgroups were compared using the log-rank test. Multivariate Cox's proportional hazard regression was used to determine the effect of all of the potential variables. Univariate regression analysis was used to evaluate the correlation between AFP level and grade of hepatitis activity. Kruskal-Wallis rank test was used to evaluate the correlation between AFP level and grade of underlying chronic liver disease. Mann-Whitney U test was used to evaluate the correlation between AFP level and recurrence. For all statistical tests, differences were considered significant at P-values <0.05. Data were analyzed with the statistical package, Dr. SPSS II® for Windows (SPSS Japan, Tokyo, Japan).

Results

Sensitivity of AFP and DCP. The sensitivity rates of pre-operative AFP and DCP for detection of HCC were 50.3% (83/165) and 61.2% (101/165), respectively. The combination of AFP and DCP increased the sensitivity to 78.8% (130/165) (Table II).

Until the end of follow-up, tumor recurrence was identified in 114 patients (69.1%). Of these, 106 (93.0%) had intrahepatic recurrence distant from the primary site, 3 (2.6%) had local tumor recurrence and 13 (11.4%) had extrahepatic recurrence (some patients had a first relapse at more than one site). The cumulative probability of overall recurrence was 46.2, 64.5 and 74.5% at 1, 2 and 3 years, respectively. The sensitivity of AFP, DCP, and their combination at the time for detecting recurrence was 55.3% (63/114), 40.4% (46/114) and 71.9% (82/114), respectively (Table II).

The time course of AFP and DCP. Among the 83 patients with a positive AFP level before operation, the value decreased and changed to negative after curative operation in 31 patients (37.3%), whereas it remained positive in 52 (62.7%). On the other hand, among the 101 patients with a positive DCP level before operation, the value changed to negative after operation in 85 (84.2%), whereas it remained positive in 16 (15.8%). The rate of negative change of AFP was significantly lower than that of DCP (P<0.001).

Fig. 1 shows the changes in perioperative AFP level in patients with (Fig. 1A and B) and without (Fig. 1C and D) recurrence. Among the patients with a positive level of AFP before operation, the number of patients whose AFP level remained positive after operation in the group with recurrence exceeded that in the group without recurrence (48/60, 80.0% vs. 4/23, 17.4%) (Fig. 1A and C), and the difference was significant (P<0.001). From a different viewpoint, among the 52 patients whose AFP level was positive both before and after operation, 48 (92.3%) experienced recurrence later (Fig. 1A and C). Among the 82 patients whose AFP level was negative before operation, only 2 (2.4%) had a positive AFP level after operation, and both of them (2/2, 100.0%) experienced recurrence (Fig. 1B and D).

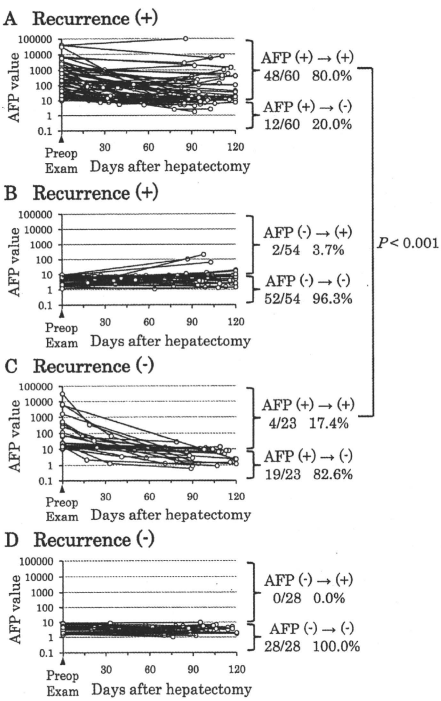

Figure 1. Pre- and postoperative serum α-fetoprotein (AFP) level. (A) Patients with recurrence and positive AFP preoperatively. (B) Patients with recurrence and negative AFP preoperatively. (C) Patients without recurrence and positive AFP preoperatively. (D) Patients without recurrence and negative AFP preoperatively. Among the patients with a positive preoperative AFP level, the number of patients whose AFP level remained positive after operation in the group with recurrence exceeded that in the group without recurrence (P<0.001). Preop exam, preoperative examination.

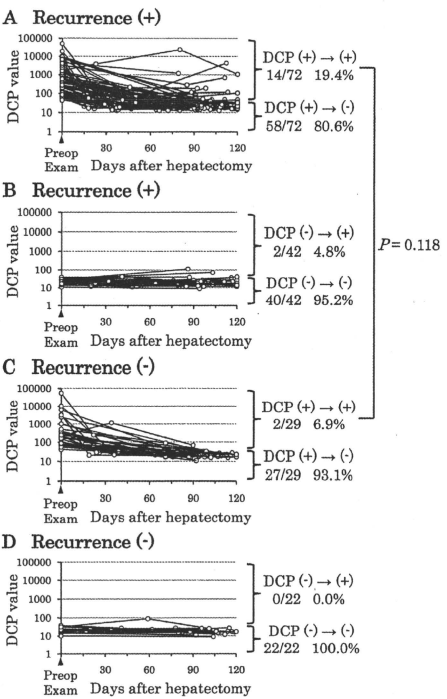

Figure 2. Pre- and postoperative serum des-γ-carboxy prothrombin (DCP) level. (A) Patients with recurrence and positive DCP preoperatively. (B) Patients with recurrence and negative DCP preoperatively. (C) Patients without recurrence and positive DCP preoperatively. (D) Patients without recurrence and negative DCP preoperatively. There was no significant difference in the number of patients whose DCP remained positive between the group with and without recurrence (P=0.118). Preop exam, preoperative examination.

Fig. 2 shows the changes in perioperative DCP level. In contrast to AFP, there was no significant difference in the number of patients whose DCP remained positive between the two groups (14/72, 19.4% vs. 2/29, 6.9%; P=0.118) (Fig. 2A and C). However, from a different viewpoint, among the 16 patients whose DCP level was positive both before and after operation, 14 (87.5%) experienced recurrence later (Fig. 2A and C). Among the 64 patients whose DCP level was negative before operation, only 2 (3.1%) had a positive DCP level after operation, and both of them (2/2, 100.0%) experienced recurrence (Fig. 2B and D).

Accuracy of minimum postoperative AFP and DCP. The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of minimum postoperative AFP, DCP and the combination of both for HCC recurrence were calculated. The sensitivity of minimum postoperative

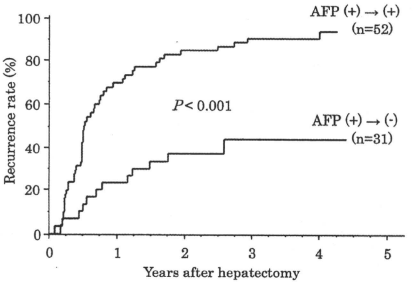

Figure 3. Overall cumulative recurrence rate curve according to minimum postoperative α-fetoprotein (AFP) level. Recurrence rate was higher in the patients whose AFP level remained positive after operation than in those whose AFP level was changed from positive to negative (P<0.001).

AFP (43.9%), DCP (14.0%) and even the combination of both (50.0%) was rather low. However, when classified by preoperative level, the minimum postoperative AFP level in patients with a positive preoperative level had higher sensitivity (80.0%) while maintaining high specificity (82.6%) and PPV (92.3%) (Table III).

Univariate and multivariate analyses to identify the risk factors for HCC recurrence. The differences in cumulative recurrence rate of patients among the various risk factors stratified were evaluated by log-rank test. Age (P=0.031), tumor occurrence (initial/recurrent) (P<0.001), minimum postoperative AFP level (P<0.001), minimum postoperative DCP level (P=0.002), tumor size (P=0.026), number of nodules (P=0.007), microscopic vascular invasion (P<0.001), intrahepatic metastasis (P<0.001) and pathological stage (P<0.001) were found to be significantly related to recurrence, whereas preoperative levels of AFP (P=0.408) and DCP (P=0.375) were not. Thereafter, multivariate analysis using Cox's proportional hazard model was performed to assess the independent importance of each variable studied. Tumor occurrence (initial/recurrent) (P<0.001), minimum postoperative AFP level (P<0.001), and intrahepatic metastasis (P=0.004) were found to be significant independent risk factors for recurrence after curative hepatectomy (Table IV). Fig. 3 shows the overall cumulative recurrence rate curve according to pre- and postoperative AFP level. When limited to the patients with a positive AFP level before operation, 52 patients whose AFP value remained positive after operation had significantly higher recurrence rate than 31 whose value changed to negative (P<0.001). Among the patients with a positive minimum postoperative AFP level, 70.4% (38/54) experienced recurrence within 1 postoperative year, 22.2% (12/54) after this time period and only 7.4% (4/54) did not experience recurrence until the end of follow-up.

Correlation between AFP level and hepatitis. To evaluate the correlation between AFP level and grade of hepatitis activity, we compared minimum postoperative AFP level with simultaneous alanine aminotransferase (ALT) level. Univariate

Table III. Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of minimum post-operative α-fetoprotein (AFP), des-γ-carboxy prothrombin (DCP), and combination of both for detection of hepatocellular carcinoma (HCC) recurrence.

	Sensitivity (%)		Specificity (%)		PPV (%)		NPV (%)	
Minimum postoperative AFP								
Overall	50/114	43.9	47/51	92.2	50/54	92.6	47/111	42.3
Regarding preoperative AFP level								
Negative	2/54	3.7	28/28	100.0	2/2	100.0	28/80	35.0
Positive	48/60	80.0	19/23	82.6	48/52	92.3	19/31	61.3
Minimum postoperative DCP								
Overall	16/114	14.0	49/51	96.1	16/18	88.9	49/147	33.3
Regarding preoperative DCP level								
Negative	2/42	4.8	22/22	100.0	2/2	100.0	22/62	35.5
Positive	14/72	19.4	27/29	93.1	14/16	87.5	27/85	31.8
Combination of both AFP and DCP								
Overall	57/114	50.0	45/51	88.2	57/63	90.5	45/102	44.1

Table IV. Cumulative recurrence rate stratified by variables using Kaplan-Meier method.

		Univariate analysis				Multivariate analysis		
		Estimated recurrence rate (%)						
	No. of patients	1-year	2-year	3-year	P-value	Odds ratio	95% CI	P-value
Age (years) (<70/≥70)	88/77	44.4/48.1	55.7/75.5	66.3/85.3	0.031	1.390	0.953-2.027	0.088
Gender (male/female)	138/27	45.0/51.9	63.0/71.4	73.9/77.1	0.691			
HBsAg (positive/negative)	36/129	44.4/46.7	60.5/65.7	72.3/75.2	0.585			
Anti-HCV (positive/negative)	103/62	45.9/46.8	66.9/60.1	78.9/66.0	0.300			
Diabetes (present/absent)	44/121	36.7/49.6	61.8/65.4	78.5/73.2	0.779			
Tumor occurrence (initial/recurrent)	108/57	35.1/67.6	55.1/83.6	67.1/90.1	<0.001	2.935	1.868-4.611	<0.001
Child-Pugh (class A/B)	150/15	44.8/60.0	64.3/66.7	74.7/75.0	0.695			
AST (IU/l) (<80/≥80)	132/33	45.6/48.5	64.9/63.6	75.2/71.7	0.861			
ALT (IU/l) (<80/≥80)	132/33	47.9/39.4	66.6/57.6	76.7/66.1	0.363			
T. Bil (mg/dl) (<1.5/≥1.5)	156/9	45.6/55.6	64.3/66.7	75.0/66.7	0.795			
PLT ($x10^3/mm^3$) (<100/≥100)	34/131	44.7/46.6	63.4/64.9	78.0/72.5	0.885			
PT (%) (<70/≥70)	31/134	32.8/49.3	59.7/65.9	71.5/75.4	0.437			
ICG-R15 (%) (<15/≥15)	75/90	46.7/45.8	62.9/65.9	76.4/74.1	0.884			
Preoperative AFP (ng/ml) (<10/≥10)	82/83	40.4/51.9	61.7/67.3	73.9/75.1	0.408			
Minimum postoperative AFP (ng/ml) (<10/≥10)	111/54	34.4/70.4	54.0/85.2	65.1/90.7	<0.001	2.331	1.574-3.453	<0.001
Preoperative DCP (mAU/ml) (<40/≥40)	64/101	47.0/45.6	64.5/64.4	68.9/79.3	0.375			
Minimum postoperative DCP (mAU/ml) (<40/≥40)	147/18	43.0/72.2	61.9/85.2	72.2/92.6	0.002	1.438	0.787-2.626	0.237
Operative procedure (<segmentectomy/≥segmentectomy)	127/38	44.3/52.6	64.8/63.2	75.0/72.4	0.600			
Tumor size (cm) (<5/≥5)	129/36	42.8/58.3	60.9/77.8	72.1/83.3	0.026	1.489	0.887-2.500	0.132
No. of nodules (solitary/multiple)	108/57	39.0/59.7	59.2/74.5	70.3/82.5	0.007	0.851	0.443-1.635	0.628
Histological differentiation (well/moderate and poor)	29/136	37.9/47.9	56.3/66.3	60.7/78.4	0.095			
Macroscopic vascular invasion (present/absent)	7/158	71.4/45.1	71.4/64.1	71.4/74.5	0.236			
Microscopic vascular invasion (present/absent)	43/122	69.8/37.9	82.1/58.2	85.1/70.7	<0.001	1.726	0.951-3.130	0.073
Intrahepatic metastasis (present/absent)	14/151	85.7/42.5	85.7/62.6	92.9/72.6	<0.001	3.066	1.425-6.594	0.004
Surgical margin (mm) (0/>0)	54/111	53.7/42.5	71.6/60.9	83.4/69.7	0.080			
Stage (UICC) (I/II, III and IV)	86/79	31.5/62.0	51.6/78.5	65.2/84.6	<0.001	1.352	0.631-2.900	0.438
Histological findings of non-cancerous lesions (cirrhosis/non-cirrhosis)	60/105	50.6/43.8	72.8/59.7	83.5/68.5	0.118			

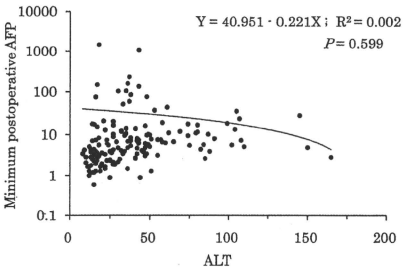

Figure 4. Correlation between minimum postoperative α-fetoprotein (AFP) and alanine aminotransferase (ALT) levels. There was no significant correlation (P=0.599).

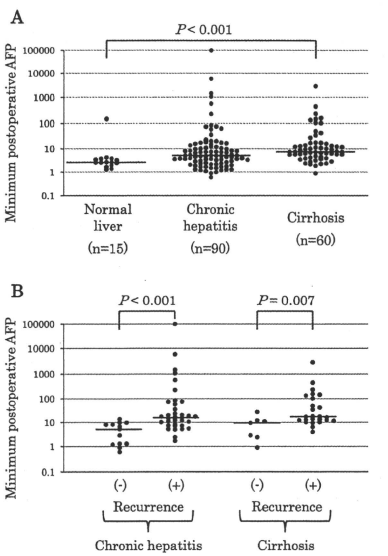

Figure 5. Correlation between minimum postoperative α-fetoprotein (AFP) and histological findings of non-cancerous lesions. (A) Patients were divided into three groups according to grade of underlying chronic liver disease. As underlying liver disease became more severe, minimum postoperative AFP level became greater (P<0.001). (B) Furthermore, patients limited to those with a positive preoperative AFP level in each group were divided into two subgroups - with and without recurrence. Minimum postoperative AFP level in patients with recurrence exceeded that in patients without recurrence in the chronic hepatitis group (P<0.001) and cirrhosis group (P=0.007). Bars are medians.

regression analysis showed no statistically significant correlation (P=0.599) (Fig. 4). To evaluate the correlation between AFP level and grade of underlying chronic liver disease, we compared minimum postoperative AFP level with histological findings of non-cancerous lesions. Fig. 5A shows minimum postoperative AFP level of the patients divided into three groups according to underlying chronic liver disease. As chronic liver disease became more severe, minimum postoperative AFP level became greater, and the difference was statistically significant by Kruskal-Wallis rank test (P<0.001). Furthermore, when patients were limited to those with a positive preoperative AFP level and the patients in each group were divided into two subgroups - those with and without recurrence, minimum postoperative AFP level of the patients with recurrence exceeded that of the patients without recurrence in the chronic hepatitis group (P<0.001) and cirrhosis group (P=0.007) (Fig. 5B).

Discussion

In this study, we demonstrated that postoperative AFP level is a useful tool for predicting HCC recurrence after curative hepatectomy. The evidence for this is that most of the patients who experienced recurrence later did not show a negative change in AFP level after curative resection. Moreover, minimum postoperative AFP level was a significant independent risk factor for recurrence. On the other hand, most of the patients who experienced recurrence later as well as those who never experienced recurrence showed a negative change in DCP level after operation, and minimum postoperative DCP was not a significant risk factor in multivariate analysis. There was no statistically significant correlation between AFP level and grade of hepatitis activity, and thus a positive level of AFP after operation might suggest a site of residual viable cancer.

Imaging modalities, including US, dynamic CT and dynamic MRI, are the gold standard for diagnosis of HCC. However, in general, since they can only detect a cancer site greater than approximately 1 cm in diameter, smaller cancer sites are missed before operation. Although intraoperative US is used to try to detect other cancer sites that have not been detected before operation, the limitations of US include its operator dependence and its poor ability to differentiate early HCC from dysplastic nodules in the cirrhotic liver. Therefore a positive level of AFP after operation might suggest a viable residual cancer site that has been undetectable by imaging modalities.

One reported problem of AFP and DCP is low sensitivity (15,16). Although measurement of two tumor markers is recommended (17-19), the sensitivity for small HCC is not yet satisfactory. However, this study showed that, when classified by preoperative level, the sensitivity of minimum postoperative AFP level was high (80.0%), whereas that of minimum postoperative DCP level was still low (19.4%). This is because AFP is superior to DCP for the diagnosis of small HCC (20). Another reported problem of AFP is low specificity because of a high false-positive rate with benign conditions such as acute and chronic active hepatitis (21-24). Several authors have demonstrated that *Lens culinaris* agglutinin-reactive α-fetoprotein (AFP-L3) can distinguish between HCC and hepatitis by detecting a sugar chain microheterogeneity (25-27). Our previous studies have demonstrated that glypican-3 (GPC3) is a novel tumor marker of HCC and is especially useful in the early stages because of its high sensitivity (28-31).

A limitation of our study is that it is difficult to determine whether an elevation of AFP is due to a residual cancer site or active hepatitis. However, our results showed no statistically significant correlation between levels of AFP and ALT, which is a well known marker of hepatitis activity (32,33). Although the histological findings of non-cancerous lesions showed a statistically significant correlation with AFP level, our results showed that patients who had higher postoperative AFP levels were most likely to experience recurrence. Moreover, not the grade of underlying chronic liver disease but postoperative AFP level was a significant risk factor for recurrence by univariate and multivariate analyses. Therefore, in most cases, a positive level of AFP after operation might mean a residual viable cancer site and not liver cirrhosis.

Generally, two different mechanisms are responsible for HCC recurrence (34). One is recurrence due to metastasis, originating from cancer cell dissemination from the primary tumor. The other is multicentric carcinogenesis of a new tumor based on underlying hepatitis or cirrhosis. However, they are not easily distinguishable (35). Instead, we distinguished between recurrence within 1 postoperative year and that after this time period as described previously (36). The result was that the majority of patients whose postoperative AFP level remained positive experienced recurrence within 1 postoperative year. Therefore, a positive level of AFP after operation suggests a site of residual viable cancer that has already occurred before operation.

In order to prevent HCC recurrence from a viable but undetectable cancer site, establishment of effective adjuvant therapy is urgently needed. We have just started a phase II clinical trial of GPC3-derived peptide vaccine for adjuvant therapy after curative operation or ablation. GPC3 is an ideal target for anticancer immunotherapy because its expression is detected specifically in most HCCs even in the early stages and is correlated with a poor outcome (37-41).

In conclusion, we have shown that minimum postoperative AFP level is an important risk factor for recurrence after curative hepatectomy. A positive level of AFP after operation might suggest a residual viable cancer site. The need for effective adjuvant therapy and close follow-up is suggested in patients with a positive postoperative AFP level. In addition, further studies will be needed to find novel useful serum markers that have better sensitivity for early detection of HCC recurrence.

Acknowledgements

This work was supported by Health and Labor Sciences Research Grants for Research on hepatitis from the Ministry of Health, Labor and Welfare.

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Identification of ß2-microgloblin as a candidate for early diagnosis of imaging-invisible hepatocellular carcinoma in patient with liver cirrhosis

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Received September 25, 2009; Accepted November 2, 2009

DOI: 10.3892/or_00000767

Abstract. Glypican-3 (GPC3) is overexpressed in hepatocellular carcinoma (HCC) but not in chronic hepatitis (CH) and liver cirrhosis (LC). We have reported the possibility of GPC3-specific cytotoxic T lymphocytes (CTLs) serving as a marker for the early diagnosis of imaging invisible HCC. In this study, to identify new early diagnostic biomarker of imaging invisible HCC, we analyzed plasma of healthy donors and patients with CH, LC and HCC using surface-enhanced laser desorption-ionaization time-of-flight mass spectrometry (SELDI-TOF-MS). The intensities of four peaks were significantly increased in HCC patients compared with healthy donors. Two of these four peaks were significantly higher in CH and LC patients with GPC3-specific CTLs than in those without. One peak (11.7 kDa) was predicted to be ß2-microglobulin (ß2-MG) by molecular mass. There was a correlation between concentration of ß2-MG by latex agglutination immunoassay in plasma and peak intensity using SELDI-TOF-MS. The 11.7 kDa protein was fractionated by gel filtration and was identified as ß2-MG by Western blot analysis. These results suggest that the level of ß2-MG in plasma from patients with CH and LC could be a useful marker for the early diagnosis of imaging invisible HCC, however further investigation is needed.

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Abbreviations: ß2-MG, ß2-microglobulin; HCC, hepatocellular carcinoma; GPC3, glypican-3; CH, chronic hepatitis; LC, liver cirrhosis; CTLs, cytotoxic T lymphocytes; AFP, α-fetoprotein; SELDI-TOF-MS, surface-enhanced laser desorption-ionaization time-of-flight mass spectrometry; LAIA, latex agglutination immunoassay; PIVKA-II, protein induced by vitamin K absence

Key words: surface-enhanced laser desorption-ionaization time-of-flight mass spectrometry, glypican-3, cytotoxic T lymphocyte, chronic hepatitis, liver cirrhosis, hepatocellular carcinoma

Introduction

Glypican-3 (GPC3) belongs to glypican family that is a group of heparan sulfate proteoglycans linked to the outer surface of cell membrane through a glycosylphosphatidylinositol anchor (1). GPC3 is overexpressed in hepatocellular carcinoma (HCC) (2) and was a useful diagnostic marker for a component HCC (3). Also, GPC3 is a useful tumor marker in early HCC (2,4,5). We have reported that GPC3 was correlated with poor prognosis in HCC (6). Furthermore, we showed the usefulness of GPC3 as a target for cancer immunotherapy (7-9). We are undertaking a phase I clinical trial of GPC3 peptide vaccine for patients with advanced HCC.

HCC is one of the most common malignant tumors worldwide (10). Patients with liver cirrhosis (LC) are at higher risk for the development of HCC (11). To date diagnostic imaging such as Computer Tomography (CT) or Magnetic Resonance Imaging (MRI) is taken as the gold standard for definitive diagnosis of HCC. Several serum markers developed for the diagnosis of HCC, including evaluation of α-feto-protein (AFP) and protein induced by vitamin K absence (PIVKA-II) (12,13). AFP and PIVKA-II is most widely used as a diagnostic serum marker for HCC, however its early diagnostic value is poor (14,15). Thus there are no available tumor markers or means for detecting invisible HCC by CT or MRI.

We have previously reported that anti-GPC3 IgG was detected in the serum of patients not only with HCC but also with chronic hepatitis (CH) and LC (16). In addition, in the same study, we reported that GPC3-specific cytotoxic T lymphocytes (CTLs) were present in the peripheral blood mononuclear cells (PBMCs) of patients not only with HCC but also with CH or LC using *ex vivo* IFN-γ enzyme-linked immunospot (ELISPOT) assay. GPC3-specific CTLs should react to the GPC3 expressing HCC cells. The detection of GPC3-specific CTLs shows the existence of GPC3 expressing

HCC cells in these CH and LC patients. This suggested that GPC3-specific CTLs could serve as a marker for the early diagnosis of imaging invisible HCC. Therefore, in this study, we tried to identify the HCC producing protein in the serum of CH and LC patients who were positive for GPC3-specific CTLs.

ProteinChip, based on surface-enhanced laser desorption-ionization time-of-flight mass spectrometry (SELDI-TOF-MS) has recently been shown to be useful in discovering biomarkers for the diagnosis of breast, liver and various other cancers (17-21). In order to establish the possibility of early diagnosis of imaging invisible HCC, we analyzed plasma from patients with CH and LC patients with or without GPC3-specific CTLs and HCC patients using ProteinChip Arrays.

Materials and methods

Plasma samples. Plasma samples were obtained from 6 patients with HCC at National Cancer Center Hospital East, 16 patients with CH or LC at Tokyo Rosai Hospital and 8 healthy volunteers after obtaining their written consent. CH and LC patients who were confirmed to be HCV-RNA (+) or HBs antigen (+) within six months prior to registration were eligible for the study. The diagnosis of CH or LC was made clinically by imaging and laboratory data. The patients had no medical history of HCC and no evidence of HCC on ultrasonography, CT or MRI conducted prior to registration. All plasma samples were stored at -80°C until analysis.

SELDI-TOF-MS analysis. For SELDI-TOF-MS analysis, we used CM10 ProteinChip (weak cation-exchange) with anionic surface chemistry. The chips were washed twice with shaking for 5 min in 150 μl binding buffer (50 mM sodium acetate, pH 4.0) per well. Plasma samples diluted 1:10 with PBS buffer and then diluted 1:10 with pH adjusted buffer. One hundred microliters of all diluted plasma samples were applied on each ProteinChips. The samples were applied in duplicate. Binding was allowed to proceed for 1 h with shaking at room temperature. The chips were then washed twice using 150 μl of binding buffer (5 min with shaking), rinsed, dried and then added 0.5 μl of a matrix solution (50% acetonitrile, 0.5% trifluoroacetic acid) to each spot. Matrix solution was repeatedly put to each spot. These ProteinChip Arrays were analyzed using a ProteinChip reader (ProteinChip Biology Systems II; Bio-Rad Laboratories, Inc., Tokyo, Japan).

High performance liquid chromatography (HPLC) analysis. Plasma samples were analyzed by the HPLC (Shimadzu, Kyoto, Japan). All samples were fractionated by size-exclusion HPLC chromatography equipped on a shodex protein KW-802.5 column (Showa Denko, Tokyo, Japan). One hundred microliters of the sample was loaded into the column: a mobile phase composed of A solution (20 mM $NaH_2PO_4{\cdot}2H_2O$ 300 mM NaCl) and B solution (20 mM $NaH_2PO_4{\cdot}2H_2O$); a flow rate of 1.0 ml/min; UV detection at 280 nm. Appropriate fractions were concentrated in Vivaspin 2 column (3 kDa cut off) (GE Healthcare, UK Ltd.). The search for retention time of ß2-MG of purified standard protein (Biocode Hycel, Liege, Belgium), fractionated using the same method for all plasma samples.

Table I. Characteristics of patients involved in this study.

	Male/ Female	Average age	HBV/HCV
GPC3-specific CTL Negative patients	5/1	60.3 (51-75)	1/5
GPC3-specific CTL Negative patients	3/3	67.7 (59-74)	2/4
HCC patients	2/4	66.1 (48-77)	2/4

Detection of ß2-microglobulin (ß2-MG) in plasma by latex agglutination immunoassay (LAIA). The concentration of ß2-MG in plasma samples was measured using a latex agglutination immunoassay (LAIA) at SRL, Tokyo, Japan. The normal range of healthy donor plasma levels of ß2-MG was 0.9-2.0 mg/l.

Western blot analysis. Plasma samples were measured by Bradford protein assay and adjusted to equal concentration for Tris-tricine SDS-polyacrylamide gel electrophoresis (PAGE). The samples in each were separated on 16.5% gels and transferred to polyvinylidene difluoride (PVDF) membrane (Millipore). The membrane was blocked by 5% milk powder in TBS-0.5% Tween-20 buffer (TBS-T) for 1 h at room temperature and then incubated with mouse anti-ß2-MG antibody (1:1000, Hokudo, Sapporo, Japan) overnight at 4°C, followed by reaction with horseradish peroxidase (HRP)-conjugated anti-mouse IgG antibody (1:20000, Jackson ImmunoResearch, USA). The signal was developed using ELC plus Western Blotting Detection Reagent (GE Healthcare, UK).

Results

Selection of the candidate protein for detecting invisible HCC using SELDI-TOF-MS. To search for novel markers for detecting invisible HCC by CT or MRI, we performed proteomic analysis using ProteinChip Array. At first, we compared the protein profiling in the plasma between 6 HCC patients and 6 healthy donors (Table I). We found four peaks (2.7, 11.7, 51.7 and 118.6 kDa) to be significantly higher in HCC patients than in healthy donors (Fig. 1). Intensity of the peaks discriminated significantly between HCC patients and healthy donors, P=0.01 (2.7 kDa), P=0.006 (11.7 kDa), P=0.04 (51.7 kDa), P=0.001 (118.6 kDa).

For further analyses, the protein expression profiles in the plasma between 6 CH, LC patients with GPC3-specific CTLs and 6 CH, LC patients without GPC3-specific CTLs were compared (Table I, Fig. 2A and B). The intensity of two peaks (11.7 and 51.7 kDa) were significantly higher than in the plasma of GPC3-specific CTLs positive patients as compared with GPC3-specific CTLs negative patients. A protein peak of 11.7 or 51.7 kDa discriminated significantly between GPC-specific CTLs negative patients and HCC patients (P=0.002 or P=0.007), as well as between GPC3-

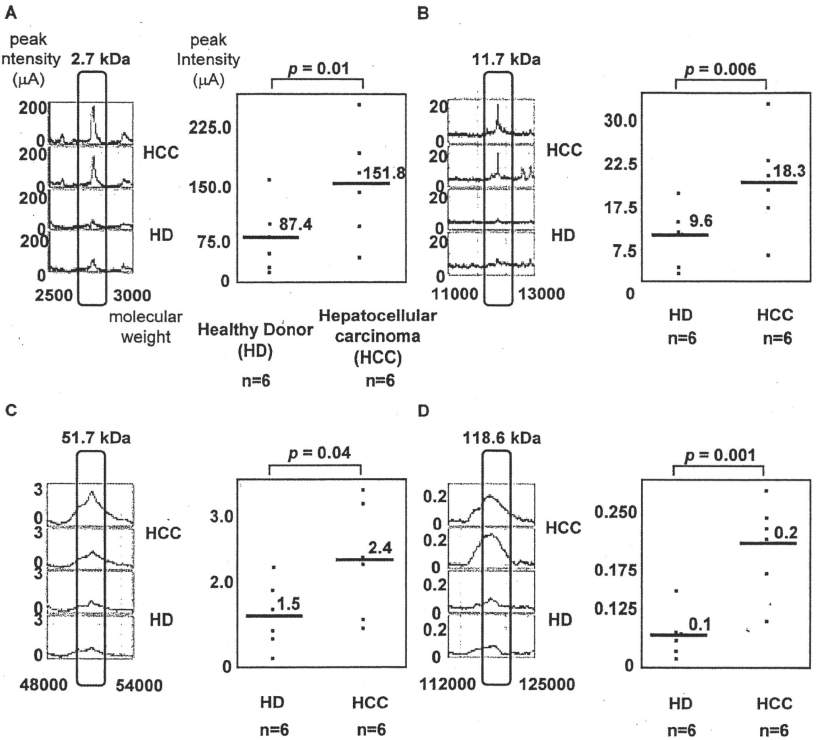

Figure 1. Comparison of the intensity of different expressed peaks in hepatocellular carcinoma (HCC) patients and healthy donors (HD). The spectra were obtained using CM10 ProteinChip (weak cation-exchange) arrays. Distribution of the intensity of different expressed peaks in plasma samples. Distribution of signal intensities for the 2.7 kDa protein (A), the 11.7 kDa protein (B), the 51.7 kDa protein (C) and the 118.6 kDa protein (D) were shown. Black bars indicate mean intensity.

Table II. Search results from SELDI-TOF-MS analysis data using ExPASy TagIdent.

Molecular weight	Entry name	Protein name	Accession no.
11731	B2-MG_HUMAN	ß2-microglobulin	P61769
11684	NRTN_HUMAN	Neurturin	Q99748
11710	KCNE3_HUMAN	Potassium voltage-gated channel subfamily E member 3	Q9Y6H6

specific CTLs positive patients and HCC (P=0.04 or P=0.02).

Prediction of ß2-microglobulin (ß2-MG) as a 11740 kDa protein using ExPASy server. To identify the 11740 kDa protein, we used the TagIdent tool from the ExPASy proteomic server (http://www.expasy.ch/tools/tagident.html). By entering the molecular mass unknown protein, this tool will search in the TrEMBL and Swiss-Plot protein database for proteins that will match with the requested molecular mass. From the results of the search from ExPASy TagIdent, we focused on ß2-MG. Peak of 11.7 kDa was predicted to be ß2-MG by molecular mass (Table II).

Identification of the ß2-microglobulin (ß2-MG). We examined the plasma level of ß2-MG in six novel donors (HD-1, 2, CH-

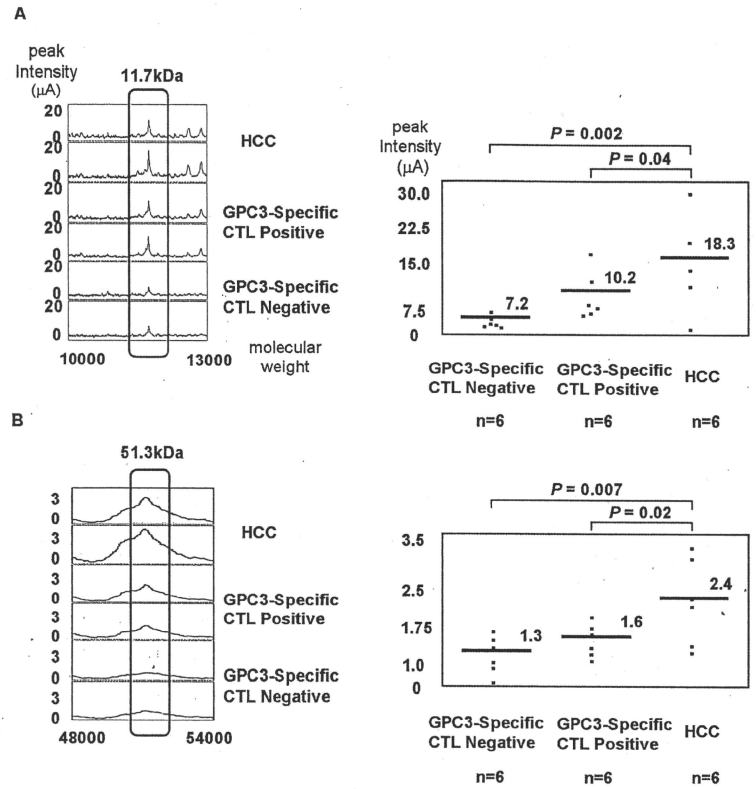

Figure 2. Comparison of the intensity of different expressed peaks in CH/LC patients who are positive for Glypican-3 (GPC3)-specific CTLs. CH/LC patients who are negative and HCC. Data were obtained using CM10 arrays. Distribution of signal intensities for the 11.7 kDa protein (A) and the 51.7 kDa protein (B) are shown.

1, 2 and LC-1, 2) by LAIA. To see whether relative peak intensity of 11.7 kDa protein in SELDI-TOF-MS analysis was correlated with plasma ß2-MG levels determined by an LAIA or not (Fig. 3). As shown in Fig. 3A and B, there was a positive correlation between the peak intensity and the concentration of ß2-MG. To further confirm our results, Western blot analysis with mouse anti-ß2-MG antibody was performed on the plasma (HD-1, 2, CH-1, 2, LC-1, 2, in Fig. 3A). As expected, a specific band at 11.7 kDa was clearly detected in plasma samples (Fig. 3C). These results suggest that 11.7 kDa band should be ß2-MG.

Fractionation of the 11.7 kDa protein peak, ß2-MG. To confirm 11.7 kDa protein as ß2-MG, the plasma was fractionated by gel filtration and HPLC. We fractionated ß2-MG standard using size-exclusion chromatography. The peak eluted for the ß2-MG standard at retention time 20.049 min (Fig. 4A). Fractions were collected at retention times from 15 to 18 min (Fr. 1), 18 to 24 min (Fr. 2: the same retention time as the fraction of ß2-MG standard), 24 to 31 min (Fr. 3), 31 to 34 min (Fr. 4) and ß2-MG standard (Fig. 4B). Western blot analysis with mouse anti-ß2-MG antibody was performed to a set of collected fractions. As expected, only a single specific band at ~12 kDa was detected with unpurified plasma, Fr. 2 and ß2-MG standard (Fig. 4C).

Discussion

SELDI-TOF-MS has been successfully applied in biomarker detection and identification in ovarian, lung, colon and

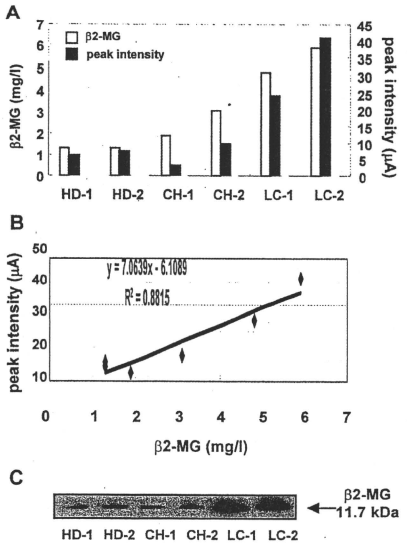

Figure3. Prediction of 11.7 kDa protein peak as ß2-microglobulin (ß2-MG). (A) Correlation between peak intensity of 11.7 kDa in healthy donors (HD)-1, 2, patients with chronic hepatitis (CH)-1, 2, liver cirrhosis (LC)-1, 2 by SELDI-TOF-MS and concentration of ß2-MG by latex agglutination immunoassay (LAIA). (B) Correlation between 11.7 kDa proteins intensity by SELDI-TOF-MS and concentration of ß2-MG by LAIA are shown. The values are well correlated. (C) Western blot analysis of ß2-MG in two pairs of healthy donors (HD) and patients (CH, LC) plasma. The band of ß2-MG is shown with an arrow.

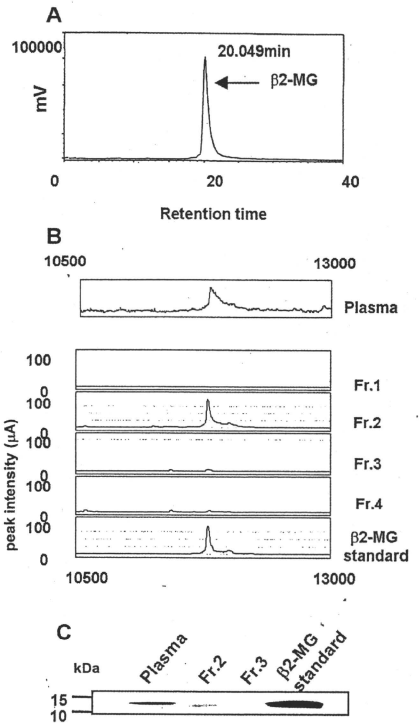

Figure 4. Fractionation of 11.7 kDa protein peak as ß2-MG. (A) High performance liquid chromatography (HPLC) of ß2-MG standard, absorbance at 280 nm. (B) Fractionation of HPLC by SELDI-TOF-MS. (C) Western blot analysis of each fractionation of HPLC and ß2-MG standard.

various cancers (22-25). In this study, in order to search for new biomarkers of CH and LC, we analyzed plasma using ProteinChip Array. We have identified ß2-MG as a new biomarker in CH and LC patients who have GPC3-specific CTLs.

ß2-MG is a non-glycosylated polypeptide composed of 99 amino acids (26). It is one of the components of major histocompatibility complex HLA class I molecules on the cell surface of all nucleated cells (27). Increased serum levels of ß2-MG also occur in a variety of multiple myeloma, lymphoma, Sjögren's syndrome and amyloid fibrils in patients receiving hemodialysis for long periods (28-30). It has been reported that the level of serum ß2-MG was elevated in patients with chronic hepatitis C, HCV-related HCC when compared to HCV-negative patients or healthy donors (31,32). However, it has not yet been reported that the detection of ß2-MG in plasma may show the invisible HCC by CT or MRI and that high ß2-MG in the plasma is a risk factor for developing HCC.

The mechanism of the increase of ß2-MG in amyloid fibrils in patients receiving hemodialysis for long periods has been elucidated, but previously the relationship between mechanism in ß2-MG and risk for the developing of HCC has not been reported.

In this study, we showed that ß2-MG in plasma increased in CH and LC patients with GPC3-specific CTLs, and suggested that the ß2-MG in plasma could be a marker to detect imaging-invisible HCC. To confirm these results, we will evaluate the correlation between level of ß2-MG and risk for developing HCC in a large-scale analysis using many plasma samples of CH and LC patients. In addition, we aim at identifying a good diagnostic marker for imaging-invisible HCC.

Acknowledgements

We thank Ms. Kaori Matsuzawa for technical assistance and Ms. Frances Gleeson for assisting in the preparation of this manuscript. This study was supported in part by Health and Labor Science Research Grants for Research on Hepatitis from the Ministry of Health, Labor and Welfare, Japan.

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Silencing of secreted protein acidic and rich in cysteine inhibits the growth of human melanoma cells with G_1 arrest induction

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(Received August 4, 2009/Revised November 25, 2009/Accepted December 7, 2009/Online publication January 20, 2010)

The overexpression of secreted protein acidic and rich in cysteine (SPARC) is associated with increased aggressiveness and poor prognosis in malignant melanoma. Its roles and underlying mechanisms on melanoma cell growth, however, are not fully clarified. To validate the potential of SPARC as a therapeutic target, we examined the effect of the knockdown of SPARC with SPARC-specific siRNA on the growth of human melanoma cell lines. SPARC siRNAs exerted a potent knockdown effect. Silencing of SPARC resulted in growth inhibition with G_1 arrest accompanied by accumulation of p21, a G_1 cyclin-dependent kinase inhibitor, in MeWo and CRL1579 cells. Moreover, the induction of p53 was observed in MeWo cells, but not in CRL1579 cells. Conditioned media containing SPARC from MeWo cells could not restore the growth of SPARC-silenced MeWo cells. This result suggests that intracellular SPARC, but not secreted SPARC, is involved in cell proliferation. In addition, silencing of SPARC induced apoptosis in MeWo and CRL1579 cells. Furthermore, when MeWo cells in which SPARC expression was transiently knocked down by SPARC siRNA were implanted in nude mice, the tumor growth was suppressed. Our findings suggest that SPARC contributes to cell growth and could be a potential target molecule for melanoma therapy. (*Cancer Sci* 2010; 101: 913–919)

The expression of secreted protein acidic and rich in cysteine (SPARC), a matricellular glycoprotein, is highly regulated during development, tissue repair, and remodeling.[1] SPARC interacts with several extracellular matrix components.[2] In addition, SPARC modulates growth factor activity, and regulates matrix metalloproteinase expression.[3–6] These reports suggest that SPARC regulates cell shape, proliferation, migration, and differentiation.

The level of SPARC expression is low in normal adult tissue, whereas this protein is overexpressed in a wide range of human cancers.[7–9] Some groups have reported that overexpression of SPARC is associated with aggressiveness and high potential of metastasis in various human cancers, including melanoma, breast, lung, esophagus, pancreas, and bladder cancers.[10–15] It has also been reported that its overexpression is related to poor prognosis in many cancers.[12,16,17] In most cancers, SPARC is produced in tumor stromal cells, such as fibroblasts and endothelial cells, rather than in cancer cells.[7,12,16] In contrast, the level of SPARC expression in melanoma and glioma cells is very high.[10,18]

Selective silencing of gene expression using siRNA has been evaluated to be not only a powerful research tool but also a potentially therapeutic approach to cancer.[19] It has been reported that silencing of SPARC directly inhibited the survival signaling pathway in glioma cells under serum reduced conditions *in vitro*.[20] Some studies using antisense RNA have showed that downregulation of SPARC abrogated a tumorigenic capacity in melanoma cells.[21–23] One of the reasons for this rejection appears to be the involvement of the antitumor activity of host polymorphonuclear cells. The underlying mechanism of SPARC on the growth of melanoma cells, however, has not been fully elucidated.

We have previously reported that the serum SPARC in melanoma patients was useful as a novel tumor marker for early diagnosis of melanoma,[24] and have shown the usefulness of SPARC as a target for cancer immunotherapy.[25] From these points of view, we hypothesized that SPARC might become a target molecule for cancer treatment, and examined whether silencing of SPARC with siRNA could influence cell growth in melanoma cells *in vitro* and *in vivo*. We found that silencing of SPARC in human melanoma cell lines induced G_1 cell cycle arrest and apoptosis. We herein report for the first time that silencing of endogenous SPARC by siRNA directly inhibits growth in melanoma cells.

Materials and Methods

Cell culture. Human melanoma cell lines MeWo, SK-MEL-28, and HMV-I were maintained in DMEM (Sigma, St Louis, MO, USA) containing 10% FBS (Hyclone, Logan, UT, USA). Human melanoma cell line CRL1579 was obtained from RIKEN Cell Bank, RIKEN BioResource Center (Tsukuba, Japan) and maintained in RPMI-1640 (Sigma) containing 10% FBS. All cells were cultured in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

RNAi and transfection. The siRNA duplexes were purchased from Qiagen (Valencia, CA, USA) (AllStars Neg. Control siRNA) and Invitrogen (Carlsbad, CA, USA) (SPARC). The siRNA sequences used were as follows: SPARC siRNA-1, 5′-AGUCACCUCUGCCACAGUUUCUUCC-3′; SPARC siRNA-2, 5′-AUACAGGGUGACCAGGACGUUCUUG-3′; and SPARC siRNA-3, 5′-AUUCUCAUGGAUCUUCUUCACCCGC-3′. Lipofectamine RNAiMax (Invitrogen) was used for the reverse transfection method following the manufacturer's protocol. For analysis of transfection efficiency, the cells were transfected with FITC-conjugated negative control siRNA (Qiagen) at 50 nM. After 24 h, the cells were analyzed using flow cytometry. Flow cytometry was carried out using a FACSCalibur (BD Biosciences, San Jose, CA, USA) and analyzed using CellQuest (BD Biosciences) and FlowJo (Tree Star, San Carlos, CA, USA) software.

Immunoblot analysis. The cell samples were lysed in appropriate amounts of lysing buffer (150 mM NaCl, 50 mM Tris [pH 7.4], 1% Nonidet P-40, 1 mM sodium orthovanadate, 1 mM EDTA, and protease inhibitor tablet [Roche Applied Sciences, Penzberg, Germany]). Protein concentration was determined

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doi: 10.1111/j.1349-7006.2009.01476.x