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## Increased immunohistochemical expression of YKL-40 in the spleen of patients with portal hypertension

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# Increased immunohistochemical expression of YKL-40 in the spleen of patients with portal hypertension

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## Abstract

YKL-40 has been identified as a growth factor in connective tissue cells and also a migration factor in vascular smooth muscle cells. To a large extent, the increase of serum YKL-40 is attributed to liver fibrosis and asthma. However, the relationship of the expression and clinical/prognostic significance of YKL-40 to the splenomegaly of patients with portal hypertension is unclear. In the present study, the expression of YKL-40 was studied by immunohistochemistry in 48 splenomegaly tissue samples from patients with portal hypertension and in 14 normal spleen specimens. All specimens were quickly stored at -80°C after resection. Primary antibodies YKL-40 (1:150 dilution, rabbit polyclonal IgG) and MMP-9 (1:200 dilution, rabbit monoclonal IgG) and antirabbit immunoglobulins (HRP K4010) were used in this study. The relationship of clinicopathologic features with YKL-40 is presented. The expression of YKL-40 indicated by increased immunochemical reactivity was significantly up-regulated in splenomegaly tissues compared to normal spleen tissues. Overexpression of YKL-40 was found in 68.8% of splenomegaly tissues and was significantly associated with Child-Pugh classification ( $P = 0.000$ ), free portal pressure (correlation coefficient = 0.499,  $P < 0.01$ ) and spleen fibrosis (correlation coefficient = 0.857,  $P < 0.01$ ). Further study showed a significant correlation between YKL-40 and MMP-9 (correlation coefficient = -0.839,  $P < 0.01$ ), indicating that YKL-40 might be an accelerator of spleen tissue remodeling by inhibiting the expression of MMP-9. In conclusion, YKL-40 is an important factor involved in the remodeling of spleen tissue of portal hypertension patients and can be used as a therapeutic target for splenomegaly.

Key words: YKL-40; MMP-9; Splenomegaly; Fibrosis; Portal hypertension; Immunohistochemistry

## Introduction

Splenomegaly is one of the complications of portal hypertension and chronic liver diseases that can influence patient morbidity and quality of life (1). The incidence of splenomegaly in cirrhosis ranges from 36 to 92% in different series (2). Especially in China, decompensated cirrhosis induced by viral hepatitis is an important cause of splenomegaly. In many cases, patients with portal hypertension simultaneously have splenomegaly and hypersplenism. They have no special symptoms related to splenomegaly but have symptoms related to hypersplenism. Occasionally, patients do not have any symptoms until they touched the enlarged spleen in the abdomen inadvertently. Splenomegaly may lead to the extrinsic compression of abdominal organs and therefore result in corresponding symptoms.

Overlarge spleens may magnify the risk for spontaneous rupture or rupture after minor trauma (1). To date, there is no specific medical therapy for splenomegaly.

YKL-40, also called human cartilage glycoprotein-39 (HC-gp39) and chitinase-3-like-1 (CHI3L1), is a heparin- and chitin-binding glycoprotein with a molecular mass of 40 kDa. It is highly conserved phylogenetically and belongs to 'mammalian chitinase-like proteins', but has no chitinase activity (3-8). The details of the physiological function of YKL-40 are unknown. It has been reported that this glycoprotein may contribute to tissue remodeling and extracellular matrix (ECM) degradation (9). An intense expression of YKL-40 protein in alveolar macrophages of idiopathic pulmonary fibrosis patients and of asthma patients demonstrates that

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it is involved in tissue remodeling and fibrosis (10,11). YKL-40 also induces the proliferation of chondrocytes and synovial cells (12). Furthermore, YKL-40 was found to induce coordination of membrane-bound receptor syndecan-1 and integrin alpha (v) beta (3) to activate an intracellular signaling cascade, including focal adhesion kinase and mitogen-activated protein kinase extracellular signal-related kinase 1/2 in endothelial cells (13). Collectively, these data suggest that YKL-40 is involved in the pathologic process of human diseases involving tissue remodeling.

Matrix metalloproteinases (MMPs) are a family of proteolytic enzymes that contain  $Zn^{2+}$ ,  $Ca^{2+}$ , and other metal ions. Accumulating evidence has indicated that MMPs play an essential role in fibrogenesis. Traditional substrates of MMPs are components of the ECM, such as collagen, laminin, and fibronectin. More than 20 enzymes have been identified as MMPs in mammals. MMP-9, an important member of the MMP family, functions in the degradation of almost all types of ECM, such as collagen I, collagen IV, collagen V, collagen VII, collagen X, collagen XI, fibronectin, elastin, and proteoglycans. Recent studies have shown that YKL-40 might play a role in fibrogenesis through MMP-9. Thus, in the present study, the relationship between expression of YKL-40 and of MMP-9 will also be explored.

Traditionally, splenomegaly in portal hypertension is thought to be a mere consequence of the rise of portal venous pressure and the increased resistance to splenic blood outflow (14), although there is a relatively weak correlation between portal venous pressure and spleen size. Recently, the role of tissue hyperplasia in congested splenomegaly has also been reported (15). Thus, we hypothesized that YKL-40 may play a role in the process of splenomegaly in portal hypertension. We also evaluated the expression of MMP-9 in splenomegaly and the relationship between YKL-40 and MMP-9 expression measured by immunohistochemistry.

## Material and Methods

### Patients and samples

Forty-eight splenomegaly specimens were obtained from consecutive patients submitted to full or partial splenectomy [33 men and 15 women with a median age of 44 years (range: 19-69)] referred to the Department of General Surgery between January 2009 and December 2009 at the Second Affiliated Hospital, Fourth Military Medical University, Xi'an, China. The diagnosis of portal hypertension was based on accepted biochemical and clinical criteria (16). In order to lower portal pressure and to alleviate clinical signs of portal hypertension (e.g., ascites, esophageal and gastric variceal bleeding, hypersplenism, splenomegaly, etc.), splenectomy was carried out with periesophagogastric devascularization or partial splenectomy was performed with a distal splenorenal shunt plus periesophagogastric devascularization after comprehensive evaluation. All

patients with portal hypertension had suffered from liver cirrhosis, 41 patients had posthepatic cirrhosis [hepatitis B (N = 35), hepatitis C (N = 6)], and 7 patients had alcoholic cirrhosis. The main morphological diagnoses as well as pertinent clinical and biochemical parameters of the patients are summarized in Table 1. During the same period, 14 normal spleen specimens were taken from patients who had suffered traumatic splenic rupture without any signs of liver disease or any other diseases. All samples used in this study were obtained with the approval of the Ethics Committee of Research in the Second Affiliated Hospital, Fourth Military Medical University, and all patients gave written informed consent to participate.

### Histological and immunohistochemical analyses

All specimens were taken from the tissues near the hilum of the spleen, which were those most directly influenced by the high portal hypertension. After 10% formalin fixation, the tissues were embedded in paraffin, cut into 5- $\mu$ m sections and routinely stained with Masson trichrome and neighboring sections were used for immunolocalization. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide for 20 min. For antigen retrieval, slides were heated in a microwave oven for 10 min in 10 mM citrate buffer, pH 6.0. Nonspecific binding was inhibited by incubation with blocking buffer at room temperature for 30 min. The sections were then incubated at 4°C overnight with primary antibodies against YKL-40 (1:150 dilution, rabbit polyclonal IgG; Quidel, USA) or against MMP-9 (1:200 dilution, rabbit monoclonal IgG; Clone ID: EP1254, Epitomics, USA). After a brief wash with phosphate-buffered saline (PBS), the sections were incubated for 30 min with a peroxidase-labeled polymer conjugated to antirabbit immunoglobulins (DAKO EnVision System/HRP K4010; Dako Cytomation, Denmark). The final detection was performed using 3,3'-diaminobenzidine (DAB) as the chromogen and 3% hematoxylin as the counterstain. Breast cancer tissue was used as positive control (immunoreactivity was noted in the tumor cells). A negative control without a primary antibody was used.

For YKL-40 and MMP-9, mainly expressed in macrophages but not in lymphocytes, expression of YKL-40/MMP-9 was tested according to the percentage of positive-stained cells. A positive result was defined when positive-stained cells were more than 10% of the total cells in one visual field at 400X magnification. The degree of fibrosis was evaluated based on the percentage of blue-stained fiber area. All images were analyzed using the Image pro plus 6.0 software (Media Cybernetics, USA).

### Statistical analysis

Statistical analysis was performed with the SPSS software (SPSS Standard version 17.0; SPSS Inc., USA). The Student *t*-test and the  $\chi^2$  test were used to evaluate the statistical significance using YKL-40 as a clinicopathologic



parameter for the patients. The Spearman  $\rho$  test was performed to evaluate correlation between YKL-40, MMP-9, free portal pressure (FPP), and degree of spleen fibrosis. P values of less than 0.05 were considered to indicate statistical significance.

## Results

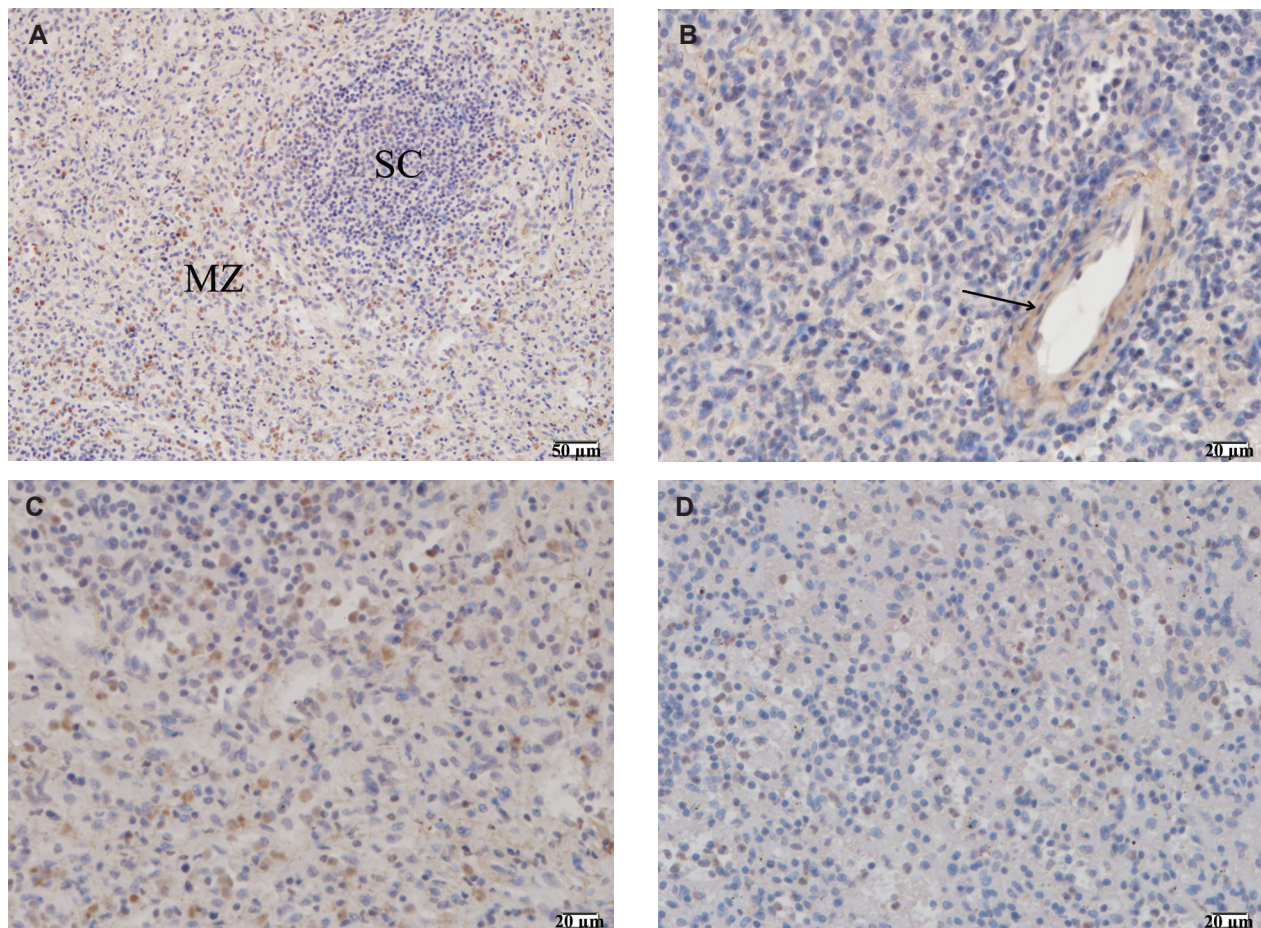
### Evaluation of YKL-40 expression in enlarged spleen

The expression of YKL-40 in 48 splenomegaly specimens and 14 normal spleen tissues was investigated by immunohistochemistry. YKL-40 was successfully detected in 33 (68.8%) of 48 splenomegaly specimens and in 3 (21.4%) of 14 normal spleen tissues. Positive expression of YKL-40 occurred in the cytoplasm. The tissues stained by YKL-40 antigen were in areas such as the splenic marginal zone and red pulp, but seldom was detected in splenic corpuscles. According to cell morphology, karyoplasmic

ratio and the location of positive-expressed cells, the positive-stained cells were macrophages. Positive-stained cells were also detected in some vascular smooth muscle cells (Figure 1).

### The relationship between overexpression of YKL-40 and the clinical features of portal hypertension

The association of YKL-40 overexpression with the clinical characteristics of the patient cohort was further evaluated and is summarized in Tables 1 and 2. The immunohistochemical expression of YKL-40 was significantly higher in the spleen of portal hypertension patients than in normal spleen. Among portal hypertension patients, there was no significant difference in red blood cells, white blood cells, platelets, serum albumin, or serum glutamic pyruvic transaminase between the YKL-40 positive-stained group and the YKL-40 negative-stained group. But serum total bilirubin (TBIL) [39.60 (21.40-74.50) vs 33.08 (15.20-51.20)],



**Figure 1.** Detection of the expression of YKL-40 by immunohistochemical staining. Positive expression (brown) of YKL-40 occurred in the cytoplasm. *A*, Expression of YKL-40 in the marginal zone (MZ) of the spleen of a patient with portal hypertension; lack of YKL-40 expression in splenic corpuscle (SC). *B*, Expression of YKL-40 in vascular smooth muscle cells (arrow). *C*, Expression of YKL-40 in spleen tissue from a patient with portal hypertension. *D*, Expression of YKL-40 in normal spleen.

serum glutamic oxalacetic transaminase (GOT) [40 (17-110) vs 30 (17-74)] were significantly higher in the YKL-40 positive-stained group than in the YKL-40 negative-stained group (Table 1). The overexpression of YKL-40 did not correlate with patient age ( $\leq 44$  years old vs  $> 44$  years old), gender, causes of cirrhosis (hepatitis B, hepatitis C, and alcoholic cirrhosis) and the severity of esophageal varices (none, slight, moderate to severe; Table 2). However, overexpression of YKL-40 was significantly related to Child-Pugh classification ( $P = 0.000$ ). When the expression of YKL-40 in different Child-Pugh classifications was compared by the chi-square method, it was found that overexpression of YKL-40 in Child B and Child C patients was higher ( $P = 0.000$ ,  $P = 0.003$ , respectively) than that in Child A patients. No difference in expression was found between Child B and in Child C patients ( $P = 0.789$ ; data not shown in Table 2). A significant correlation between expression of YKL-40 and FPP was also obtained by the Spearman  $\rho$  test (correlation coefficient = 0.499,  $P < 0.01$ ; Figure 2).

#### Correlation of the overexpression of YKL-40 and MMP-9 in fibrosis

It has been reported that YKL-40 is able to activate the release of MMP-9 in alveolar macrophages from patients with chronic obstructive pulmonary disease (17) and from the human monocytic cell line THP-1 (18) when these cells were exposed to YKL-40. But a recent study (19) showed that YKL-40 in infiltrating macrophages inhibits MMP-1 activity and accelerates fibril formation in the vicinity of macrophages to trap macrophages in adipose tissue. To determine whether YKL-40 is able to mediate the activation of MMP-9 in spleen of patients with portal hypertension, expression of MMP-9 and its relationship with YKL-40 were investigated in spleen tissue with portal hypertension by immunohistochemistry. Positive-stained cells were also located mainly in the red pulp and marginal zone but scarcely in the splenic corpuscle. Interestingly, expression of MMP-9 in normal spleen was indeed higher than in enlarged spleen ( $37.39 \pm 0.75$  vs  $25.11 \pm 0.83\%$ ;  $P < 0.01$ ), and a significant correlation was found between YKL-40 and MMP-9 (correlation coefficient = -0.839,  $P < 0.01$ ; Figure 3).

Histomorphological analysis was carried out to detect the presence of fibrosis in the spleen by Masson trichrome staining, and a considerable amount of fibrosis extending to

the entire splenic parenchyma was found in portal hypertension patients compared to normal persons ( $17.07 \pm 5.03$  vs  $5.11 \pm 0.73\%$ ,  $P < 0.01$ ). Additionally, the Spearman  $\rho$  test showed that expression of YKL-40 was correlated with the fibrosis area of the spleen (correlation coefficient = 0.857,  $P < 0.01$ ; Figure 4).

**Table 1.** Clinical and biochemical characteristics of the patients with portal hypertension.

	YKL-40 positive-stained (N = 33)	YKL-40 negative-stained (N = 15)
Age (years)	45 (22-69)	40 (19-58)
Red blood cells ( $10^{12}/L$ )	3.59 (2.64-5.02)	3.41 (2.57-3.90)
White blood cells ( $10^9/L$ )	1.68 (0.10-7.04)	2.02 (0.77-3.11)
Platelets ( $10^9/L$ )	43 (20-119)	50 (11-94)
Serum albumin (g/L)	31.7 (25.8-41.8)	33.6 (27-38.7)
Serum TBIL ( $\mu M$ )	39.6 (21.4-74.5)	33.1 (15.2-51.2)*
Serum GPT (U/L)	27 (9-75)	21 (11-61)
Serum GOT (U/L)	40 (17-110)	30 (17-74)*

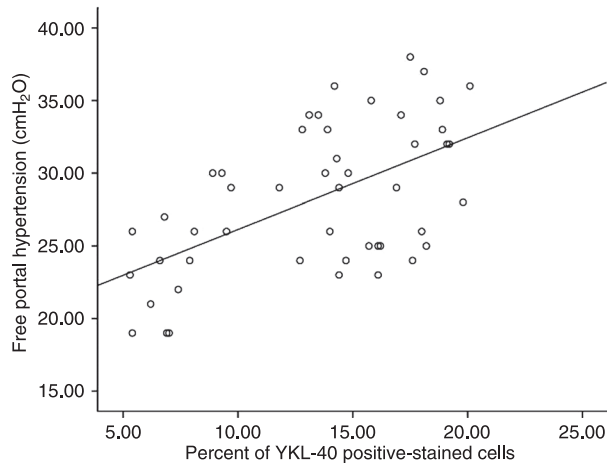
Data are reported as median (range). TBIL = total bilirubin; GPT = glutamic-pyruvic transaminase; GOT = glutamic-oxalacetic transaminase. \* $P < 0.05$  compared to YKL-40 positive-stained group (Student *t*-test).

**Table 2.** Clinical significance of YKL-40 overexpression in portal hypertension.

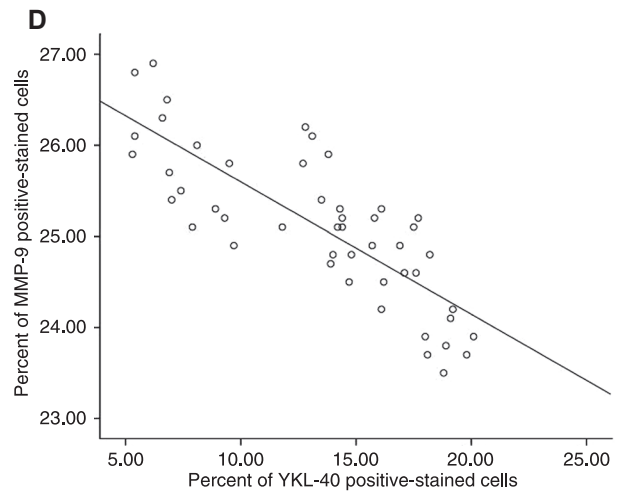
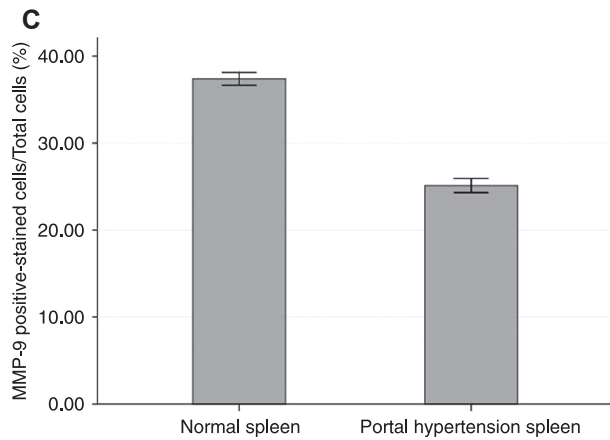
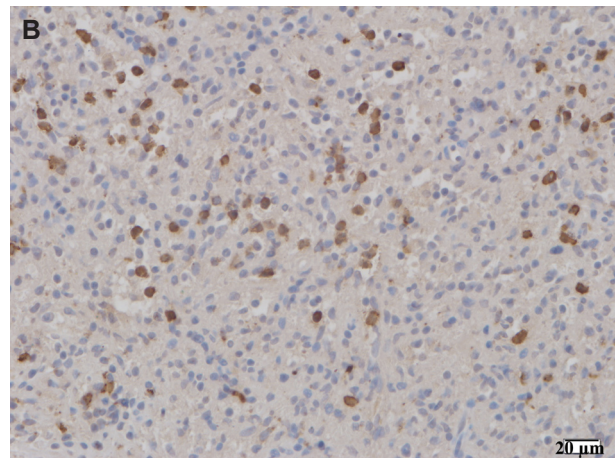
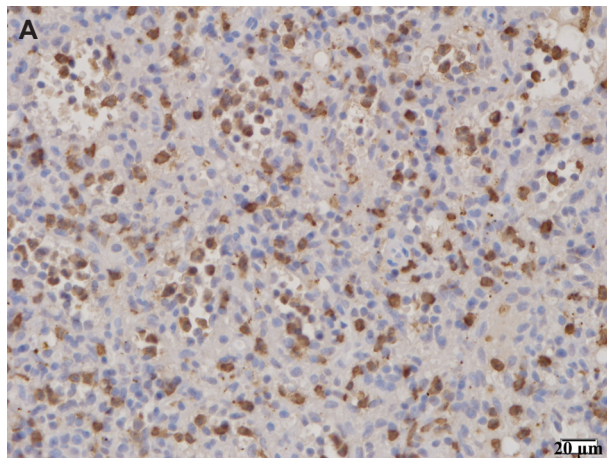
Clinicopathologic features	Total cases	Overexpression of YKL-40
Normal spleen	14	3/14 (21.4%)*
No. of patients	48	33/48 (68.8%)
Age (years)		
$\leq 44$	27	16/27 (59.3%)
$> 44$	21	17/21 (81.0%)
Gender		
Male	33	24/33 (72.7%)
Female	15	9/15 (60.0%)
Cause of cirrhosis		
Hepatitis B	35	24/35 (68.6%)
Hepatitis C	6	4/6 (66.7%)
Alcoholic cirrhosis	7	5/7 (71.4%)
Severity of esophageal varices		
None	3	1/3 (33.3%)
Slight	9	6/9 (66.7%)
Moderate/Severe	36	26/36 (72.2%)
Child-Pugh classification		
Child A	14	3/14 (21.4%)*
Child B	26	22/26 (84.6%)*
Child C	8	8/8 (100%)*

\* $P < 0.05$  compared to patients with portal hypertension or degrees of Child-Pugh classification ( $\chi^2$  test).

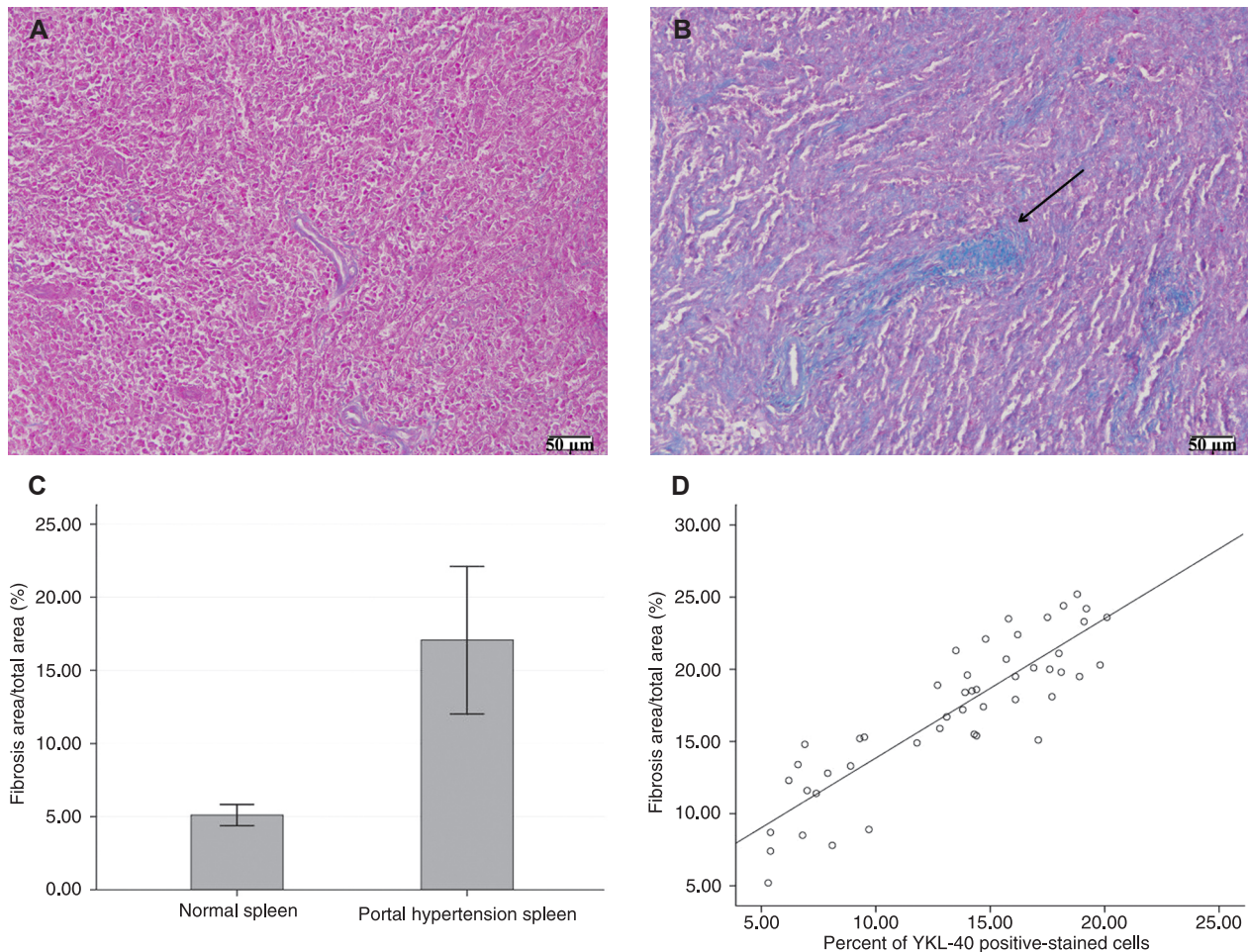




**Figure 2.** Correlation between the expression of YKL-40 and free portal pressure.  $R = 0.499$ ;  $P < 0.01$  were obtained with the Spearman  $\rho$  test.



**Figure 3.** Expression of metalloproteinase-9 (MMP-9) in the spleen. Positive expression (brown) of MMP-9 occurred in the cytoplasm. *A*, Normal spleen; *B*, spleen from a patient with portal hypertension; *C*, comparison of MMP-9 between a normal spleen and spleen tissue from a patient with portal hypertension. Data are reported as means  $\pm$  SD. *D*, Correlation between YKL-40 and MMP-9 expressed in spleen tissue from a patient with portal hypertension ( $R = -0.839$ ,  $P < 0.01$ ; Spearman  $\rho$  test).



**Figure 4.** Detection of fiber in the spleen by Masson trichrome staining. *A*, Normal spleen. *B*, Spleen from a patient with portal hypertension. The arrow indicates blue staining of the fiber. *C*, Comparison of the amount of fiber between a normal spleen and spleen tissue from a patient with portal hypertension. *D*, Correlation between expression of YKL-40 and fiber in spleen tissue from a patient with portal hypertension ( $R = 0.857$ ,  $P < 0.01$ ; Spearman  $\rho$  test).

## Discussion

YKL-40 was first detected by Johansen et al. (3) as an ECM glycoprotein in a human osteosarcoma MG63 cell line. So far, expression of YKL-40 has been detected in some kinds of cancer cells (20,21), macrophages (22,23), fetal cells in the human embryo (24), tumor-associated macrophages (25), in the late differentiation of neutrophil activation (26), in cartilage cells (4,27), in the differentiation of vascular smooth muscle cells (28,29), and fibroblast-like chondrocytes (26,30) but not in lymphocytes. As to tissue remodeling, YKL-40 has been shown to be expressed by a variety of cells during chronic inflammatory conditions (31) and its serum concentration is associated with the risk and severity of asthma (10,32), acute myocardial infarction (33), rheumatoid arthritis (34), and atherosclerosis (35). Functionally, YKL-40 is secreted by activated macrophages during

early and late stages of differentiation *in vitro* (6,22). *In vivo*, YKL-40 mRNA is found in a subpopulation of macrophages in tissues with inflammation and matrix remodeling (36). Johansen et al. (9) and Berres et al. (37) have reported that serum YKL-40 is associated with severity of liver fibrosis and can be considered to be a noninvasive fibrosis marker. Our study initially showed that the expression of YKL-40 was significantly higher in the spleen of patients with portal hypertension than that in the spleen of normal people. In the spleen, YKL-40 positive-stained cells were detected mainly in the marginal zone and red pulp in which large macrophages were located, and scarcely found in the splenic corpuscle where most lymphocytes were located. Based on cell morphology, karyoplasmic ratio and the location of the positive-expressed cells, they were identified as macrophages. This result is consistent with previous studies. Nishikawa and Millis (29) have reported that YKL-40 is a

novel adhesion and migration factor for vascular cells. In our study, positive-stained vascular muscle cells were also detected in some individually proliferating vessels. However, positive-stained cells were not found in most proliferating vessels, although smooth muscle proliferation was their common feature. This demonstrated that YKL-40 may be engaged in vascular remodeling when there is a direct irritation of the hyperdynamic circulation or fibrosis-promoting factors in the blood circulation, but YKL-40 may not be the main factor stimulating smooth muscle proliferation in the splenomegaly of portal hypertension. Because the spleen specimens were taken from patients suffering from cirrhosis of different origins, the causes of cirrhosis were also evaluated. However, no difference was found ( $P = 0.982$ ) demonstrating that there was no relationship between overexpression of YKL-40 and causes of cirrhosis.

Normally, fibers support the structure of the spleen and represent the blood spleen barrier. However, fiber proliferates largely in splenomegaly of portal hypertension. Thus, it will break the normal structure of the spleen and block blood flow into the spleen. In addition, fiber proliferation limits further expansion of the sinusoids to accept more blood and stimulates compensatory angiogenesis. More importantly, fiber proliferation increases the mechanical damage to blood cells and clearance of blood cells by macrophage, which will lead to hypersplenism. Thus, we explored whether splenomegaly associated with portal hypertension was driven by fibrogenesis. Our results suggested that this could be the case and statistical analysis also indicated that overexpression of YKL-40 was significantly associated with the degree of fibrosis in the spleen associated with portal hypertension. This may well demonstrate that YKL-40 is an important factor contributing to the process of spleen fibrosis. The Spearman  $\rho$  test also showed that overexpression of YKL-40 was correlated with FPP and Child-Pugh classification. It has been reported (38) that if the spleen remained in the artificial system of portal hypertension, fibrosis of the spleen would be increased and its function would disappear gradually, but if it migrated to the iliac vessels with normal blood pressure, the fibrosis could be gradually reduced. This indicates that portal hypertension might indirectly be the immediate cause of spleen fibrosis. The correlation between YKL-40 and FPP showed that the direct stimulation of portal pressure may be one of the factors involved in the overexpression of YKL-40. As to the correlation with Child-Pugh classification, the more seriously liver function was impaired, the more YKL-40 was expressed. Serum TBIL and GOT could reflect liver func-

tion and injury to the hepatocytes. The difference in serum GOT/serum TBIL between the YKL-40 positive-expressed group and YKL-40 negative-expressed group may also indirectly demonstrate the influence of liver function on the overexpression of YKL-40 in splenomegaly. Although this may not be the full reason for the result of our study, it can be hypothesized that with the deterioration of liver function, some factors causing liver fibrosis may flow into the spleen with the blood circulation and stimulate the expression of YKL-40.

MMPs are important factors in the ECM. Under normal physiological conditions, they are thought to be of great importance in the maintenance of connective tissue integrity. MMP-9, a member of the MMP family, was reported to have proteolytic activity against connective tissue proteins, and has been suggested to be important in the connective tissue remodeling processes associated with atherogenesis and plaque rupture (39). Theoretically, we hypothesized that YKL-40 promoted fibrosis in the spleen by inhibiting expression of MMP-9 in the process of portal hypertension. Interestingly, the results agreed with this hypothesis and led us to believe that inhibition of MMP-9 by YKL-40 may be the key molecular mechanism involved in the development of fibrosis in splenomegaly derived from portal hypertension. However, further studies are needed.

In our study, we only investigated the function of YKL-40 in fibrogenesis, one aspect of splenomegaly caused by portal hypertension. Although we detected overexpression of YKL-40 in some individual vascular smooth muscle cells, the effects of YKL-40 on angiogenesis are not yet fully known. As to hypersplenism, the lack of differences in red blood cells, white blood cells and platelets between the YKL-40 positive-stained group and the YKL-40 negative-stained group may indicate that YKL-40 has no impact on this condition, but more studies are needed.

In conclusion, YKL-40 is an important factor in the spleen fibrogenesis occurring in portal hypertension. Moreover, combined with the routine treatment of portal hypertension (modifying liver function, lowering free portal hypertension, etc.), development and application of YKL-40 inhibitors or YKL-40 antibody may be an effective target of therapy for splenomegaly. Further investigations are in progress.

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