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Quantitative fibrosis parameters highly predict esophageal-gastro varices in primary biliary cirrhosis

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Abstract. – OBJECTIVE: Esophageal-gastro Varices (EGV) may develop in any histological stages of primary biliary cirrhosis (PBC). We aim to establish and validate quantitative fibrosis (qFibrosis) parameters in portal, septal and fibrillar areas as ideal predictors of EGV in PBC patients.

PATIENTS AND METHODS: PBC patients with liver biopsy, esophagogastroscopy and Second Harmonic Generation (SHG)/Two-photon Excited Fluorescence (TPEF) microscopy images were retrospectively enrolled in this study. qFibrosis parameters in portal, septal and fibrillar areas were acquired by computer-assisted SHG/TPEF imaging system. Independent predictor was identified using multivariate logistic regression analysis.

RESULTS: Among the forty-nine PBC patients with qFibrosis images, twenty-nine PBC patients with both esophagogastroscopy data and qFibrosis data were selected out for EGV prognosis analysis and 44.8% (13/29) of them had EGV. The qFibrosis parameters of collagen percentage and number of crosslink in fibrillar area, short/long/thin strings number and length/width of the strings in septa area were associated with EGV ($p < 0.05$). Multivariate logistic analysis showed that the collagen percentage in fibrillar area ≥ 3.6% was an independent factor to predict EGV (odds ratio 6.9; 95% confidence interval 1.6-27.4). The area under receiver operating characteristic (ROC), diagnostic sensitivity and specificity was 0.9, 100% and 75% respectively.

CONCLUSIONS: Collagen percentage in the fibrillar area as an independent predictor can highly predict EGV in PBC patients.

Key Words:
Quantitative fibrosis, Primary biliary cirrhosis, Esophageal-gastro varices.

Introduction

Primary biliary cirrhosis (PBC) is a chronic autoimmune liver disease characterized by destructive cholangitis, destruction of interlobular bile ducts with progressive fibrosis and cirrhosis leading to complications such as portal hypertension and liver failure[1,2].

Esophageal-gastro varices (EGV) as an ominous sign is present when the threshold of portal hypertension reaches 10-12 mmHg, affecting approximately 50% of the cirrhotic patients[3,4]. Current guidelines recommend endoscopy screening for all patients with cirrhosis to identify those who should receive prophylactic treatment[5]. For the PBC patients, EGV may develop in cirrhotic and pre-cirrhotic stages with a prevalence of 30%[6]. More than 10% of PBC patients have EGV in the early stage. About 40% of these patients have esophageal varices bleeding witthe hin three years. The five-year survival rate is 63%, compared with 91% of the patients without EGV[7]. Most of the non-invasive markers can only predict EGV in the late stage of liver fibrosis[2,7-10], but EGV can occur in any stages of PBC. Up to now, no consistent and reproducible marker for EGV prediction in PBC has been identified[7].

Several hypotheses about the pathogenesis of portal hypertension based on histological evaluation have been reported[11-14]. It may be related to granulomatous inflammation which results in portal venous branch compression, perisinusoidal fibrosis and nodular regenerative hyperplasia. Fibrosis severity is one of the very important parts of pathological change in PBC, leading to development of portal hypertension and subsequent

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complications including ruptured esophageal, splenomegaly and ascites[15]. So it would be very important to determine the relationship of liver fibrosis parameters with EGV.

Recently, Second Harmonic Generation (SHG) and Two-Photon Excitation Fluorescence (TPEF) (SHG/TPEF) microscopy imaging system without staining have been developed, reducing the inter-observer and intra-observer variation of the traditional histological evaluation system[16,17]. The SHG/TPEF microscopy system provides a quantitative measurement of the collagen level and preservation of the morphological information in images, which can detect subtle and dynamic changes of different collagen. Combining pathology-relevant collagen architectural features with automated computer-aided image analysis tools, the fully quantitative fibrosis (qFibrosis) parameters including portal, septal and fibrillar parameters were acquired.

The aim of this study is to detect and validate qFibrosis parameters as ideal predictors of EGV in PBC patients.

Patients and Methods

Patients and Data Collection

We reviewed the clinical records of PBC patients from March 2009 to November 2014 in Beijing Friendship Hospital, Capital Medical University. Patients who had liver biopsy specimen with the following clinical data were included in this study: liver histological diagnosis description, esophagogastroscopy findings, antimitochondrial antibody, liver biochemistry examination, hematological parameters and ultrasound examination at the time of liver biopsy. The presence of EGV was confirmed by esophagogastroscopy in this study.

The diagnosis of PBC was defined by at least two of the following criteria according to the 2009 PBC practice guideline from the American Association for the Study of Liver Diseases (AASLD)[2]: (1) Biochemical evidence of cholestasis based on alkaline phosphatase elevation; (2) Presence of antimitochondrial antibody; (3) Absence of biliary obstruction by ultrasound, computed tomography, or cholangiography; (4) Histologic evidence of destructive cholangitis and destruction of interlobular bile ducts. Histological stages of PBC were evaluated according to the Ludwig et al criteria[18]. Stages I and II were considered as early stage, III as mid-stage and IV as late stage. The Mayo risk score was calculated using the Mayo Natural History Model for Primary Biliary Cirrhosis at the time of liver biopsy.

Exclusion criteria: (1) Coexistent with other liver diseases, such as chronic viral hepatitis B/C, autoimmune liver disease, alcoholic or non-alcoholic fatty liver disease, primary sclerosing cholangitis or inherited metabolic liver disease; (2) Spontaneous encephalopathy, variceal hemorrhage or diuretic-resistant ascites; (3) Other co-existing conditions such as cancer, renal failure or severe congestive heart failure.

Among the forty-nine PBC patients with qFibrosis images, twenty-nine PBC patients with both esophagogastroscopy data and qFibrosis data were selected out for EGV prognosis analysis. Patients were separated into two groups: EGV group and non-EGV group.

SHG/TPEF Microscopy Imaging and Processing

All of the twenty-nine liver biopsy specimens were routinely fixed in formalin, embedded by paraffin and sectioned at 5 μm thickness. Then the slices were dewaxed without staining for imaging by the system of SHG/TPEF microscopy (HistoIndex, Genesis 200®, Singapore). Collagen was visualized by SHG microscopy and hepatocyte morphology was acquired by the TPEF microscopy. We used the full scan system to acquire the images. Images were acquired at 20× magnification with 512 × 512 pixel resolutions. Image processing and analysis (threshold and SHG scoring) were routinely performed with quantitative features of the SHG/TPEF image acquired by the computer-aided system as previously reported[16,17,19]. There are one hundred collagen morphological features in this study. These features were divided into three groups: the collagen proportions including the total, aggregated and distributed collagen percentages; collagen string properties such as the thickness, length, width and so on; the ratios of the different collagen string types. Slices of each specimen were then stained by Masson Trichrome for histological comparison with the SHG/TPEF images.

Informed written consents were obtained from all patients before enrollment in this trial and the study was approved by the Research Ethics Committee of Beijing Friendship Hospital, Capital Medical School.

Statistical Analysis

Statistical analysis for continuous data was performed by Student's *t*-test and statistical analysis for categorical data was performed by χ^2 or Fischer exact test. EGV predictors were determined using univariate and multivariate logistic regression analysis. The optimal cut-off value of EGV predictor was determined at the maximum of total sensitivity and specificity. The diagnostic performance of the independent predictor was determined by sensitivity, specificity, positive and negative predictive values and receiver operating characteristic (ROC) curves. The area under the ROC curve shows the ability to identify patients with EGV from those without EGV.

Results

Clinical Characteristics of PBC Patients with and without Esophageal-Gastro Varices

Among the 49 PBC patients with qFibrosis data, 29 PBC patients with both esophagogastroscopy data and qFibrosis data were selected out for EGV prognosis analysis. 93.1% (27/29) of them were female and the median age was 50 (46-56 years old). Patients who had EGV were older in age (53.0 *vs*. 47.5 years old). Overall, 44.8% (13/29) of our patients had EGV on esophagogastroscopy with 27.3% (3/11) in early stage, 28.5% (2/7) in middle stage and 72.7% (8/11) in late stage. Clinical characteristics of PBC patients with and without EGV were listed in Table I.

There was asignificant difference in serum albumin, platelet count and Mayo risk score in the two groups. Patients with EGV had lower albumin levels (32.3 *vs*. 38.1 g/L), lower platelet counts (79.0 *vs*. 168.5 thousands/mm^3) and higher Mayo risk score (5.6 *vs*. 4.4) compared with patients without EGV ($p < 0.05$). The antimitochondrial antibody M2 subtype (AMA-M2) and total bilirubin status were higher in EGV group (68.7 *vs*. 50.5 IU/L and 32.5 *vs*.18.3 □mol/L), but the difference is not significant ($p > 0.05$). Furthermore, univariate and multivariate analysis of clinical characters showed these two parameters has no correlation with EGV. Gender, other liver biochemistries, hematological parameters and prothrombin time were similar between the two groups.

Morphological Liver Fibrosis Features Visualized by Masson and SHG/TPEF Microscopy

Masson's trichrome staining was conventionally performed to assess liver fibrosis, with the collagen dyed in blue-green and hepatocytes stained in red. In our study, collagen fibers in Masson staining were stained in light blue (Figure 1A), which is not so bright as in SHG/TPEF image which uses green as positive staining signals. In SHG/TPEF image, collagen (green) was detected by SHG signal and hepatocyte morphology (red) was detected by the TPEF signal. SHG/TPEF image without staining faithfully matches the traditional pathological image stained with Masson trichrome and also can pro-

Table I. Clinical parameters of PBC patients with and without EGV.

Clinical parameters	EGV (n = 13)	Non-EGV (n = 16)	*p*-value
Gender (female/total)	92.3% (12/13)	93.8% (15/16)	0.741
Age (year)	53.0 (47.5-59.0)	47.5 (44.8-54.5)	0.123
AMA (positive/total)	23.0% (3/13)	25% (4/16)	0.625
IgG (mg/dl)	1600.0 (1350.0-2000.0)	1695.9 (1057.0-2130.0)	0.930
IgM (mg/dl)	373.0 (217.0-479.0)	355.0 (276.2-509.0)	0.569
ALT (U/L)	65.0 (44.0-90.5)	62.0 (36.3-123.0)	0.843
AST (U/L)	70.0 (55.0-117.0)	74.0 (43.8-114.8)	0.742
ALP (U/L)	278.0 (167.0-377.0)	265.0 (141.0-397.5)	0.769
GGT (U/L)	235.0 (116.0-344.0)	334.0 (176.8-751.0)	0.069
ALB (g/L)	32.3 (25.4-37.2)	37.7 (32.8-41.3)	0.017
TBIL (μmol/L)	32.5 (14.5-56.4)	18.3 (12.7-33.1)	0.125
PLT ($\times 10^9$/L)	79.0 (63.5-162.5)	168.5 (97.0-305.0)	0.011
INR (ratio)	1.1 (1.0-1.1)	1.0 (0.9-1.0)	0.112
Mayo Risk Sore	5.6 (4.7-8.7)	4.4 (3.9-5.3)	0.010

Data are presented as median (*interquartile range*). AMA indicates antimitochondrial antibody; IgG, Immunoglobulin G; IgM, Immunoglobulin M; ALT, alanine aminotransferase; AST, aspertate aminotransferase; ALP alkaline phosphatase; GGT, gamma-glutamyl transpeptidase; ALB, albumin; TBIL, total bilirubin; PLT, Platelets; INR, International Normalized Ratio.

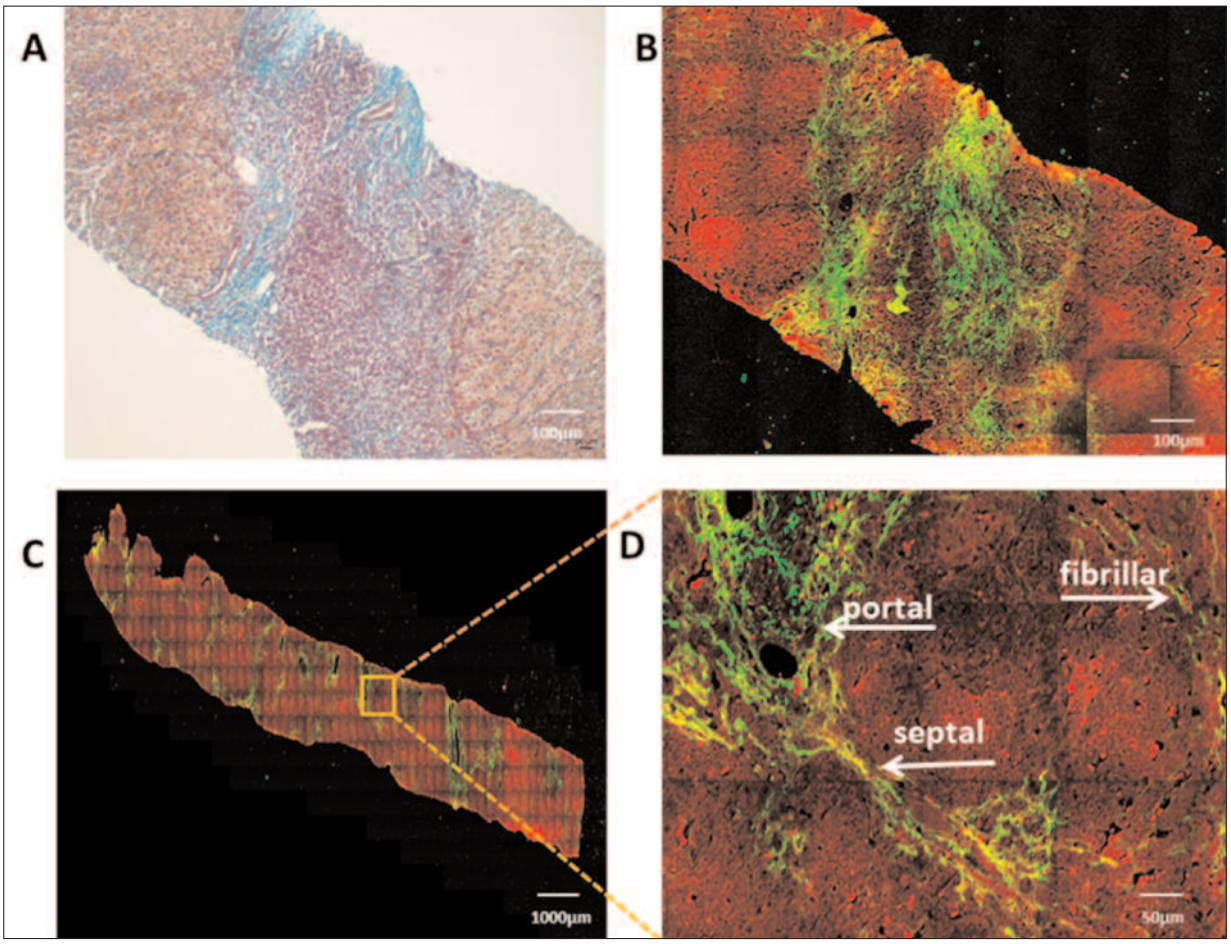

Figure 1. SHG/TPEF imaging faithfully matches image of Masson's trichrome staining. ***A,*** Liver biopsy slice stained with Masson's trichrome. Collagen fibers are stained in blue, and hepatocytes are stained in red. ***B,*** SHG/TPEF microscopy images without staining of liver biopsy slice. Collagen (*green*) was detected by SHG signal, and hepatocyte morphology (*red*) was observed with TPEF signal. ***C,*** SHG/TPEF microscopy image of a complete liver biopsy slice. ***D,*** Morphological features of collagen in different area: collagen in portal tracts (Portal), a fibrous bridge connects a portal tract (Septa), and fibrosis surround hepatic and sinusoid cells (Fibrillar).

vide more image information of liver fibrosis for further quantitative analysis (Figure 1B).

In complete liver biopsy slice images using SHG/TPEF microscopy, fibrosis is shown as bright green area. Amplification of one part of the complete slice could clearly show morphological features of collagen in different areas, including the collagen in portal tracts (Portal area), fibrous bridge (Septal area) and fibrosis around hepatic and sinusoid cells (Fibrillar area) (Figure 1 C and D). The area of these bright green imaging was quantified by feature parameters in three categories: the collagen proportion including the total, aggregated and distributed collagen percentages; collagen strings properties such as the thickness, length, width and so on; the ratios of the different collagen string types.

Predictors of Esophageal-Gastro Varices

On univariate analysis, qFibrosis parameters of collagen percentage and number of crosslinks in the fibrillar area, short/ long/ thin strings number and length /width of the strings in the septal area were associated with EGV ($p < 0.05$). None of the parameters in the portal area were associated with EGV. Multivariate logistic analysis showed that the collagen percentage in the fibrillar area was an independent factor to predict EGV (odds ratio is 7.2; 95% confidence interval is 1.7-29.3) (Table II).

Univariate analysis of clinical data showed serum albumin, platelet count, and Mayo risk score was associated with the presence of EGV ($p < 0.05$). Multivariate analysis of clinical characters and qFibrosis factors associated

Table II. Univariate and multivariate analysis of qFibrosis factors associated with presence of EGV.

qFibrosis parameters	Univariable OR (95% CI)	*p*-value	Multivariable (final model) OR (95% CI)	*p*-value
Total area				
Distributed collagen percentage	2.6 (1.0-6.5)	0.049		
Number of strings	1.3 (1.1-1.5)	0.008		
Number of short strings	1.4 (1.1-1.9)	0.006		
Number of long strings	1.6 (1.0-2.4)	0.036		
Number of thin strings	2.1 (1.1-3.7)	0.016		
String length	1.0 (1.0-1.0)	0.020		
String width	1.0 (1.0-1.0)	0.027		
String eccentricity	1.3 (1.0-1.6)	0.008		
Septal area				
Number of short strings in septal area	1.5 (1.1-2.1)	0.017		
Number of long strings in septal area	1.8 (1.2-5.9)	0.019		
Number of thin strings in septal area	2.7 (1.2-5.9)	0.016		
String length in septal area	1.0 (1.0-1.0)	0.022		
String width in septal area	1.0 (1.0-1.0)	0.026		
Fibrillar area				
Collagen percentage in fibrillar area	4.3 (1.4-13.0)	0.009	7.2 (1.7-29.3)	0.006
Number of crosslinks in fibrillar area	1.0 (1.0-1.1)	0.019		

with the presence of EGV showed that the collagen percentage in the fibrillar area was an independent factor to predict EGV (odds ratio is 6.9; 95% confidence interval is 1.6-27.4) (Table III).

The ROC curve for the presence of EGV showed that the collagen percentage in fibrillar area of 3.6% was the best cut-off value based on the high sensitivity (100%), specificity (75%), positive predictive value (PPV, 92.9%), negative predictive value (NPV, 100%) and accuracy (86.2%) with area under the curve (AUC) of 0.9 (Figure 2).

Discussion

EGV has a strong correlation with progression of PBC and it can occur in any stages of PBC. Although AASLD guidelines recommend that PBC patients should be screened of EGV by esophagogastroscopy when the platelet count is below 14,000/mm^3 or Mayo risk score is over 4.1, there are many other criteria for EGV screening in PBC, most of which were based on biochemical markers.

To our knowledge, this study is the first one of fully quantitative assessment of liver fibrosis and

Table III. Univariate and multivariate analysis of clinical characters and qFibrosis factors associated with presence of EGV.

qFibrosis parameters	Univariable OR (95% CI)	*p*-value	Multivariable (final model) OR (95% CI)	*p*-value
Clinical				
ALB (g/L)	1.3 (1.1-1.5)	0.008		
PLT	1.0 (1.0-1.0)	0.036		
Mayo Risk Score	2.9 (1.1-7.4)	0.026		
qFibrosis				
Number of short strings in septal area	1.5 (1.1-2.1)	0.017		
Number of long strings in septal area	1.8 (1.2-5.9)	0.019		
Number of thin strings in septal area	2.7 (1.2-5.9)	0.016		
String length in septal area	1.0 (1.0-1.0)	0.022		
String width in septal area	1.0 (1.0-1.0)	0.026		
Collagen percentage in fibrillar area	4.3 (1.4-13.0)	0.009	6.9 (1.6-27.4)	0.008
Number of crosslinks in fibrillar area	1.0 (1.0-1.1)	0.019		

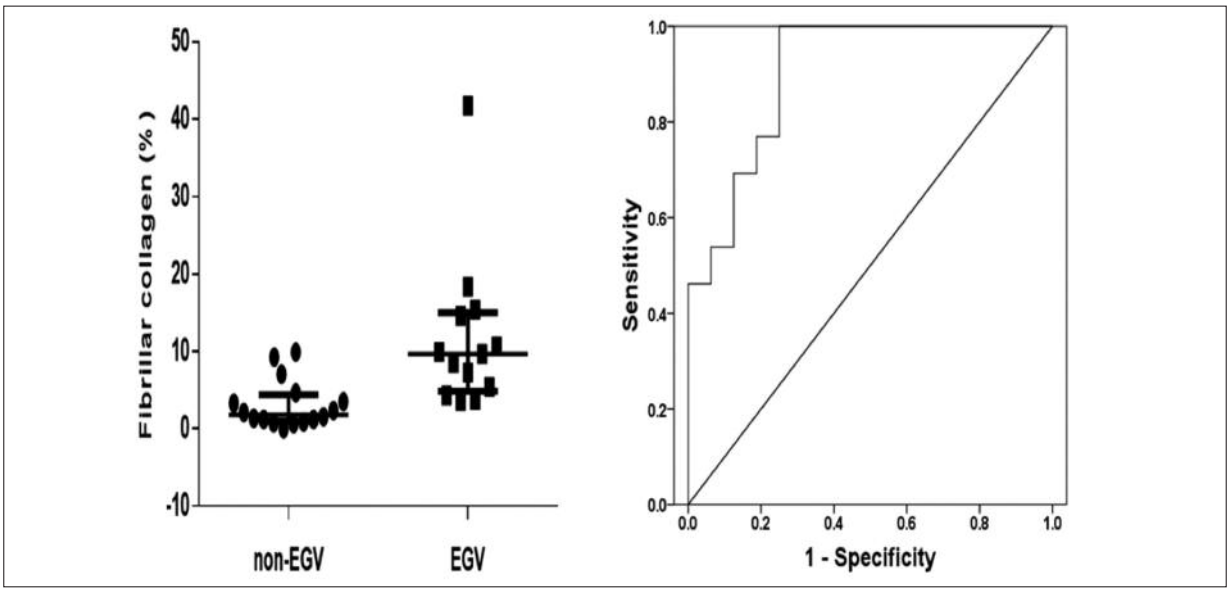

Figure 2. qFibrosis parameters of collagen percentage in fibrillar area as an independent factor can highly predict Esophageal-Gastro Varices (EGV) in PBC regardless of histological stage. The collagen percentage in fibrillar area is significantly different between EGV and non-EGV group ($p < 0.001$). As an independent predictor of EGV in PBC, the optimal cut-off value was 3.6%. The diagnostic sensitivity and specificity was 100% and 75% respectively. The area under curve (AUC) of ROC, the positive predictive value, negative predictive value and accuracy was 0.9 (95% CI: 0.8-1.0), 92.9%, 100% and 86.2% respectively.

to investigate its relationship with EGV in a large cohort of PBC patients. In this study, we found 44.8% of the PBC patients had EGV, with 27.3% of them happened in the early histological stage. We explained EGV presence in PBC from a quantitatively pathological point of view. Quantitative fibrosis parameters of collagen percentage in the fibrillar area can highly predict EGV in PBC patients as an independent predictor. We noticed that even in the early stage of the PBC, most of our SHG/TPEF images showed significant fibrillar collagen quantified around the perisinusoidal area, which demonstrated that dynamic changes of the collagen in the fibrillar area may play an important role in the pathological process of EGV in PBC patients. Additionally, our results proved the hypothesis that perisinusoidal fibrosis is one of the important pathogenesis mechanism of EGV, especially in the early stage of PBC.

Our study also showed short/ long/ thin strings number and length /width of the strings in the septal area were associated with EGV. Hyperplastic ('regenerative') nodules results in the diversion of blood from the hepatocyte and flowing of the blood through shunting vessels along fibrous septa, which were more common in the patients with EGV than those without EGV[20].

No qFibrosis parameters of portal area in EGV patients in this study was significantly higher than that of patients without EGV, which is in consistent with the results of a study from Kew et al[20] who claimed that marked portal fibrosis without nodules was equally common in the patients with and without varices and the degree of portal fibrosis was not significantly different.

Inflammation evaluation is important for PBC histological stages. Significant portal tract inflammation is one of the reasons for portal hypertension in the early stage of PBC[11]. By comparing the SHG/TPEF and hematoxylin-eosin staining images, we found the bright red dots surround the hepatocyte may present a special form of inflammation, which needs to be proved and quantified in the future study.

Conclusions

Our study confirms that the presence of EGV can occur in both pre-cirrhotic and cirrhotic stages of PBC. Collagen percentage and number of crosslink in the fibrillar area, short/ long/ thin strings number and length /width of the strings in the septal area were significantly associated with the presence of EGV. The collagen percentage in

the fibrillar area can highly predict EGV in PBC patients as an independent factor. Our findings provide important quantitative of pathological information for EGV prediction in PBC using the newly developed SHG/TPEF microscopy imaging system.

Consent

Written informed consent was obtained from the patients for the publication of this report and any accompanying images.

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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