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Research

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Peritumoral ductular reaction can be a prognostic factor for intrahepatic cholangiocellular carcinoma

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Running Title

Peritumor DR and ICC

Key words: peritumoral, ductular reaction, prognosis, intrahepatic cholangiocellular carcinoma

Abstract

Background: Peritumoral ductular reaction (DR) was reported to be related to the prognosis of combined hepatocellular-cholangiocarcinoma and hepatocellular carcinoma. Non-mucin-producing intrahepatic cholangiocellular carcinoma (ICC), may be derived from small bile duct cells or liver progenitor cells (LPCs). However, whether peritumoral DR is also related to non-mucin-producing ICCs needs to be investigated.

Methods: Forty-seven patients with non-mucin-producing ICC were eventually included in the study. Clinicopathological variables were

collected. Immunohistochemical analysis and immunofluorescence staining for cytokeratin 19, proliferating cell nuclear antigen, and α-smooth muscle actin were performed in tumor and peritumor liver tissues.

Results: Peritumoral DR is significantly correlated with local inflammation and fibrosis. Patients with significant peritumoral DR had high recurrence rate (81.8% vs 56.0%, $P = 0.058$) and poor overall and disease-free survival time ($P = 0.01$ and $P = 0.03$, respectively). Significant peritumoral DR showed high proliferation activity of LPC/cholangiocytes and abundant background extracellular matrix.

Conclusions: Patients with non-mucin-producing ICC having significant peritumoral DR had a poor prognosis. Peritumoral DR could be a prognostic factor for ICC; however, the mechanism should be further investigated.

Keywords: Intrahepatic cholangiocellular carcinoma, non-mucin producing, ductular reaction, prognostic factor

Background

Cholangiocarcinoma (CCA) is highly malignant with a 5-year survival rate of 0%–10% [1]. CCA can be divided mainly into extrahepatic cholangiocarcinoma (ECC) and intrahepatic cholangiocarcinoma (ICC) based on the anatomic features [2]. ICC accounts for approximately 10% of primary liver cancer, and the incidence continues to increase in recent

years [3]. ICC can also be divided into two categories: mucin producing and non-mucin producing, which have different pathological characteristics and origin. Although ICC is thought to develop from the intrahepatic bile duct, at least some ICCs, especially non-mucin-producing ICCs, may be derived from small bile duct cells or liver progenitor cells (LPCs) [4,5]. In combined hepatocellular-cholangiocarcinoma (CHC), which may originate from bipotential LPC due to their dual characteristics of cholangiocytes and hepatocytes, background LPC was reported to be a prognostic factor after resection due to the origin of tumor cells [6]. Ductular reaction (DR) consisting of LPCs or small cholangiocytes represents hepatic or biliary regeneration in the liver. Peritumoral DR consisting of LPCs or small cholangiocytes is related to the prognosis of CHC and hepatocellular carcinoma (HCC) [6,7]. However, whether similar finding can also be observed in non-mucin-producing ICC needs to be investigated.

In the present study, patients with non-mucin-producing ICC were included, and the extent of peritumoral DR was evaluated to explore whether a relationship existed between DR and prognosis of ICC as well as its potential mechanism.

Methods

Patients and specimens

From January 2004 to December 2016, 47 patients with ICC, who underwent curative surgery in Shanghai General Hospital, Shanghai Jiaotong University School of Medicine were included in the study. Informed consent was obtained from each patient under a protocol approved by the ethics committee of each hospital. The tumor stage was determined according to the 2009 UICC TNM classification system [8]. Data on laboratory parameters including serum albumin, alanine aminotransferase, aspartate aminotransferase, total bilirubin, and alpha-fetoprotein were collected. The tumor and peritumor (<2 cm away from the tumor) tissues from each patient and the available nontumor tissues (>2 cm away from the tumor) from 30 patients were paraffin embedded.

Histological and immunohistochemical analysis

Liver tissues were fixed in 4% formaldehyde and embedded in paraffin. Sections (4 μm thick) were stained with hematoxylin and eosin stain. According to the Scheuer scoring system, the inflammation and fibrosis of the peritumor tissues were blindly scored by two experienced pathologists [9]. For immunohistochemical analysis, after a phosphate-buffered saline wash, the sections were transferred into 10mM sodium citrate buffer (pH 6.0), and antigen unmasking was performed in a microwave. After cooling down, the sections were incubated with peroxidase blocking reagent (Dako, Hamburg, Germany) for 1 h and then

stained overnight at 4°C with the following primary antibodies: anti-cytokeratin 19 (CK19) (Dako), 1:200; anti-proliferating cell nuclear antigen (PCNA) (Santa Cruz Biotechnology, USA), 1:200. The sections were developed with diaminobenzidine for 5 min. The DR grade was evaluated according to the following standard: 0, no or minimal DR around a few portal tracts and septa; 1, focal DR around most portal tracts/septa; 2, continuous DR around <30% of portal tracts/septa; 3, continuous DR around 30% – 50% of portal tracts/septa; and 4, continuous DR around more than 50% of portal tracts/septa (Fig. 1). For quantitative analysis, mean values of immunoreactive cells by counting the three fields including the portal or septal area at 200×magnification were randomly obtained. The proliferation index (PI) was calculated as the ratio between the number of $PCNA^+$ cells and the total number of reactive ductular cells or tumor cells.

Double-fluorescence immunostaining

Double-fluorescence immunostaining of formalin-fixed, paraffin-embedded tissue was performed using a sequential fluorescence method as previously described [10]. Alexa488 or Alexa647-conjugated goat antirabbit antibody (Invitrogen) was used as secondary antibody. Immunofluorescence was observed using Olympus IX-71 inverted microscope.

Statistical analysis

Data were analyzed using SPSS version 23.0 for Windows (SPSS Inc, Chicago, IL, USA) and presented as means and standard deviations (±SD). Student *t* tests were used to compare the continuous quantitative data. A two-tailed Wilcoxon signed-rank test was used to compare ranked variables. The correlation between the degree of DR and clinicopathological variables was determined by Spearman or Pearson correlation as appropriate. A *P* value <0.05 was considered statistically significant.

Results

Clinicopathological and follow-up data

Forty-seven patients (30 men and 17 women) were eventually included in the present study, with a mean age of 58.4 ± 11.0 years. Nine patients had lymph node invasion, and 5 patients had distant metastasis at the time of diagnosis. After a follow-up period of 25.7 ± 19.1 months, 32 patients (68.1%) developed intrahepatic recurrence after surgery (Table 1).

Peritumoral DR was related to local inflammation and fibrosis

The correlation analysis showed that peritumoral DR was significantly

correlated with local inflammation and fibrosis ($r = 0.357$, $P = 0.008$ and $r = 0.742$, $P < 0.001$, respectively) (Fig. 2a). Due to the different definition of background DR, the DR grade in peritumor (<2 cm away from the tumor) and nontumor (>2 cm away from the tumor) areas was also compared in 30 patients whose liver tissues from both sites were available. The results demonstrated a small difference in local inflammation and fibrosis between them, but with no statistical significance. The DR grade in nontumor area paralleled with that in peritumoral area ($r = 0.713$, $P < 0.001$) (Fig. 2b). These results indicated a similar extent of DR and local environment between peritumor and nontumor areas.

Peritumoral DR was related to the prognosis of ICC

According to the grade of peritumoral DR, patients with ICC were divided into two groups: mild peritumoral DR (grades 1 and 2) ($n = 25$) and significant DR (grades 3 and 4) ($n = 22$). Age, gender composition, TNM stages, and tumor differentiation were not significantly different between these two groups. However, the recurrence in the significant DR group was much higher than that in the mild DR group (81.8% vs 56.0%, $P = 0.058$) (Table 1). The survival analysis showed that patients with significant peritumoral DR had poor overall and disease-free survival ($P = 0.01$and $P = 0.03$, respectively) (Fig. 3).

Different proliferation of peritumoral ductular cells

According to the previous description, PI was used to mark the extent of proliferation (Fig. 4a). The significant peritumoral DR group showed a higher PI trend of ductular cells compared with the mild peritumoral DR group but failed to achieve statistical significance due to high variation (0.43 ± 0.29 vs 0.28 ± 0.31, $P = 0.172$). The percentage of high PI (>50%) ductular cells was also higher in the significant peritumoral DR group (44.44% vs 30.77%, $P < 0.01$). Undoubtedly, the tumor cells showed much higher PI compared with the other two groups (Fig. 4b).

Different grade of peritumor DR was related to different microenvironments

ICC is a kind of tumor with abundant extracellular matrix (ECM), which plays an indispensable role in tumor progression. Double-fluorescence immunostaining showed the α-smooth muscle actin (α-SMA)-positive fibrosis background and CK19-positive ductular and tumor cells. The results demonstrated more abundant ECM and α-SMA-positive vessels in peritumoral areas in the significant DR group than in the mild DR group, which was similar to that in the tumor (Fig. 5a). The correlation analysis showed that the DR grade was positively related to the portal/septal α-SMA level (Fig. 5b).

Discussion

ICC can pathologically be divided into two categories: mucin producing and non-mucin producing. The former originates from large intrahepatic bile ducts and has pathological features similar to those of ECC, while the latter is considered to be derived from LPC or small bile duct cells [4,5]. ICC and HCC share some common risk factors, providing evidence that some ICC might originate from bipotential LPC. A recent meta-analysis showed that cirrhosis and hepatitis B or hepatitis C virus (HCV) infection was a potential risk factor with the odds ratio value of 22.92, 5.1, and 4.8 respectively [11]. Another study showed that HCV infection and cirrhosis were the main risk factors for ICC [12]. On the contrary, liver fibrosis and inflammation were also main factors influencing the extent of DR in chronic liver diseases [13-15]. Therefore, it was speculated that peritumoral DR might be related to ICC due to common influencing factors and cell origin.

For CHC derived from LPC, the active peritumoral LPC was considered to be related to recurrence after resection [6]. Peritumoral DR was also correlated with the prognosis in HCC [7]. However, the relationship between peritumoral DR and prognosis of ICC is still not elucidated. Because non-mucin-producing ICC may be derived from small cholangiocytes or LPCs, which are the main source of DR, their relationship was studied. In the present study, the peritumoral DR was

closely related to local liver inflammation and fibrosis, just like in chronic liver disease. Also, a similar extent of DR and liver inflammation or fibrosis was found in peritumor and nontumor areas (<2 or >2 cm from the tumor), indicating that such microenvironment is not just located in peritumoral areas.

According to the grade of peritumoral DR, patients with ICC were divided into two groups: mild DR and significant DR groups. The clinical and pathological variables were compared between the two groups, and the results showed that the latter had a significantly higher recurrence rate compared with the former. The survival analysis showed that patients with significant peritumoral DR had significant poor overall and disease-free survival time. Hence, it could be concluded that peritumoral DR was related to non-mucin-producing ICC and could be a prognostic factor.

The mechanism underlying the relationship between peritumor DR and ICC is still not clear. A study demonstrating the correlation of peritumor DR with the prognosis of CHC speculated that peritumoral LPC probably provided the field effect and led to the development of tumor [6]. The present study also showed that peritumoral DR was related to ICC occurrence and poor prognosis. However, it is still hard to elucidate whether the activated peritumor LPCs/cholangiocytes could cause tumor occurrence. Immunostaining by PCNA showed that the proliferation activity of LPC was significantly enhanced in the significant DR group

than in the mild DR group. The abundant ECM is the characteristic of ICC, and the tumor-related macrophages and fibroblasts located in the ECM are related to poor prognosis. Immunostaining by α -SMA also demonstrated that significantly abundant ECM and vessels were accompanied by obvious DR, indicating that significant peritumoral DR might share similar microenvironment with ICC. Therefore, significant peritumoral DR shared some characteristics with ICC, and it was possible that peritumoral DR also offered “field effect” or was the potential source of recurrent ICC.

Conclusions

The present study showed that patients with ICC having significant peritumoral DR had a poor prognosis. Significant peritumoral DR has a high proliferation activity of LPCs/cholangiocytes and abundant background ECM, similar to ICC. Although it is unclear whether activated peritumoral LPCs/cholangiocytes could lead to tumor occurrence or only be the result of fibrosis, which is also a risk factor of ICC, the results suggested that peritumoral DR could be a prognostic factor for ICC. However, the mechanism should be further investigated.

Authors’ contributions

Conceived and designed the study: XBC, LGL, XJW. Collected and

analyzed the data: ZYS, JBX, JJW. Interpreted the results and wrote the paper: XBC, ZYS, JBX, JJW. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

Not applicable.

Consent for publication

Not applicable.

Ethics approval and consent to participate

The clinical study was approved by the Ethics Committee of Shanghai General Hospital, Shanghai Jiaotong University School of Medicine. Informed consent was obtained from each participator.

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Figure Legends

Fig. 1 Representative figures showing different grades of peritumoral DR

indicated by immunohistochemical staining for CK19 in ICC (scale bar = 200 μ m).

Fig. 2 The relationship among inflammation, fibrosis and DR grade in peritumoral and nontumor tissues. **a** Grade of DR was closely correlated with local inflammation and fibrosis in peritumoral liver tissues. **b** Comparison of local inflammation, fibrosis, and DR grade between peritumoral and nontumor areas.

Fig. 3 Kaplan–Meier analysis of the overall and disease-free survival of patients with ICC having different grades of DR.

Fig. 4 PI in the different peritumoral DR groups and tumor group. **a** CK19/PCNA positive expression in the mild and significant peritumoral DR group and tumor group. **b** The significant peritumoral DR group indicated a higher PI trend of ductular cells compared with the mild peritumoral DR group, and tumor group showed much higher PI compared with the other two groups. [PI = number of PCNA-positive (arrow)/total number of reactive ductular/tumor cells].

Fig. 5 Microenvironment in the different peritumoral DR groups and tumor group. **a** The significant DR group had more abundant ECM and α-SMA-positive vessels in peritumoral areas than in the mild DR group, and tumor group had similar microenvironment with the significant DR group. **b** The correlation analysis illustrated that the DR grade was positively related to the portal/septal α-SMA level.

Figures

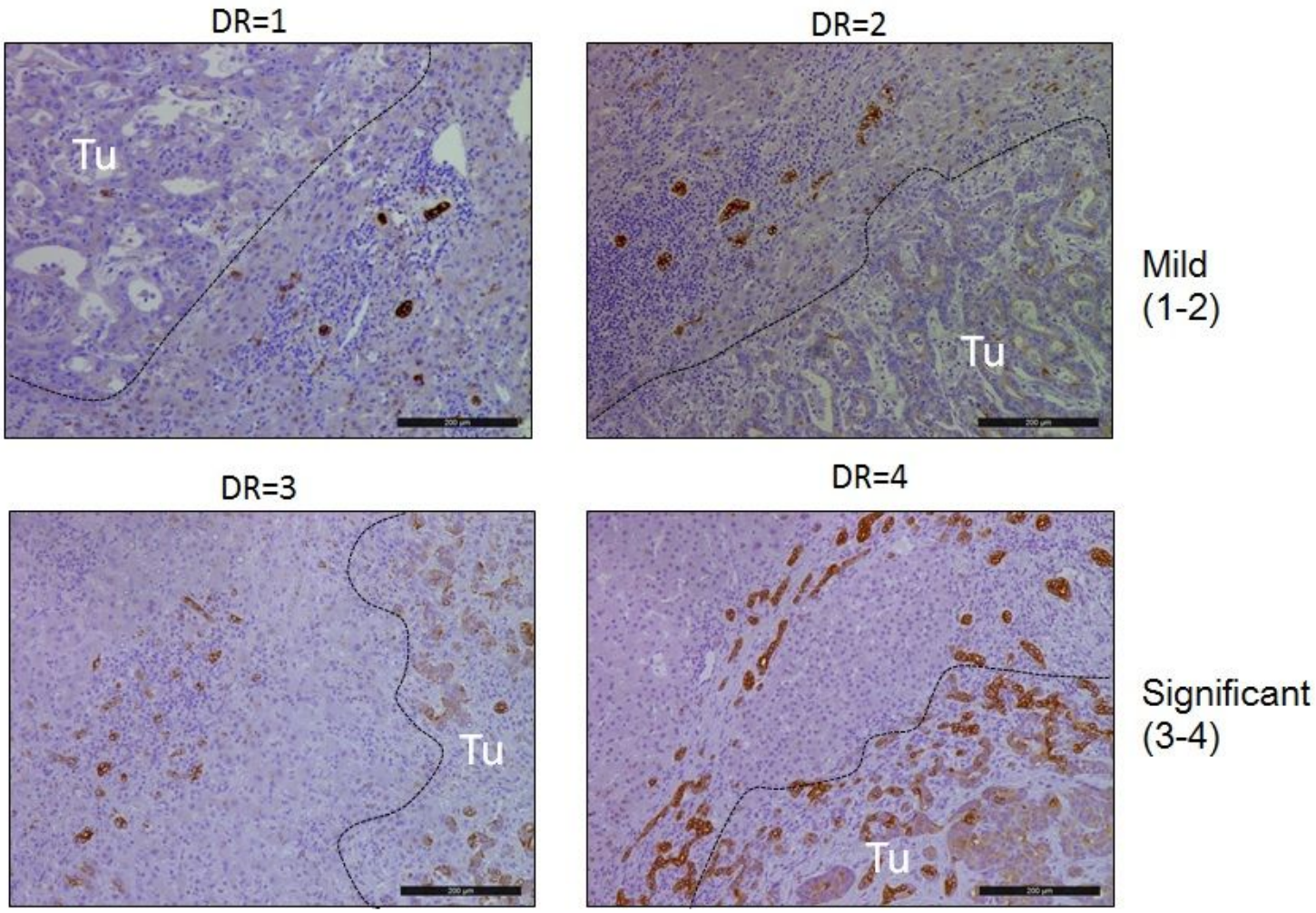

Figure 1

Representative figures showing different grades of peritumoral DR indicated by immunohistochemical staining for CK19 in ICC (scale bar = 200 μm).

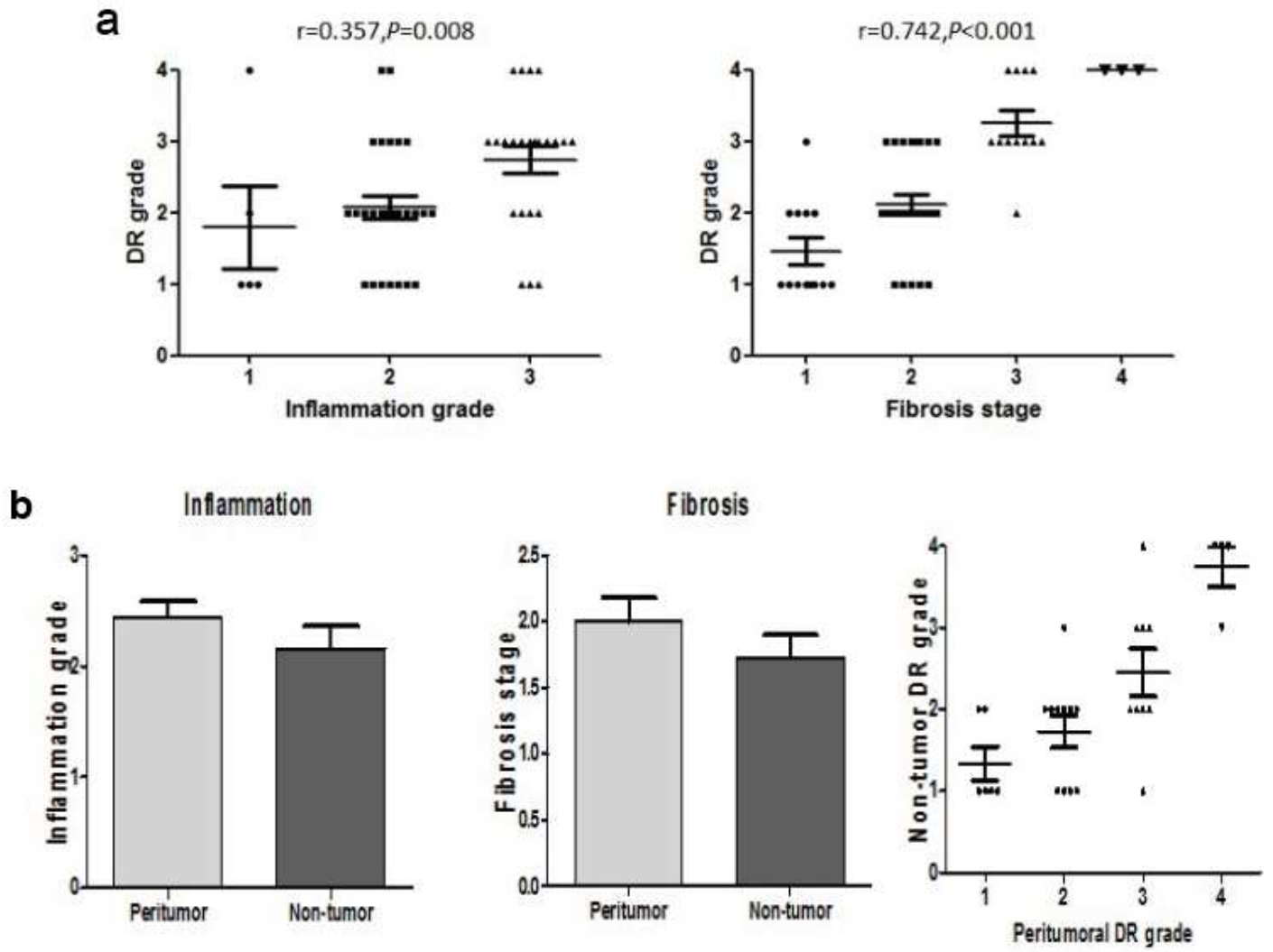

Figure 2

The relationship among inflammation, fibrosis and DR grade in peritumoral and nontumor tissues. a Grade of DR was closely correlated with local inflammation and fibrosis in peritumoral liver tissues. b Comparison of local inflammation, fibrosis, and DR grade between peritumoral and nontumor areas.

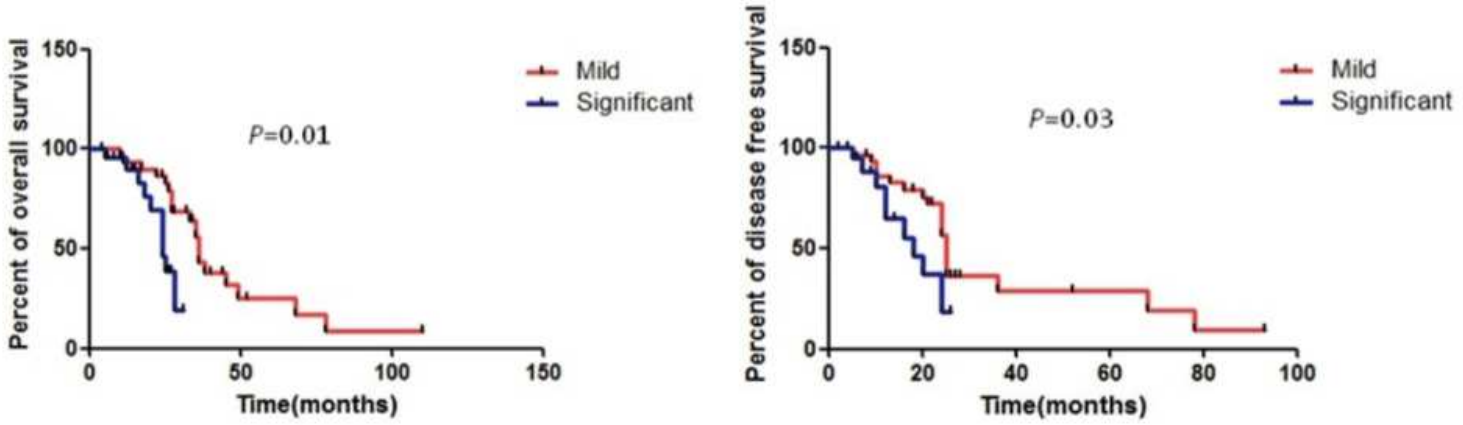

Figure 3

Kaplan–Meier analysis of the overall and disease-free survival of patients with ICC having different grades of DR.

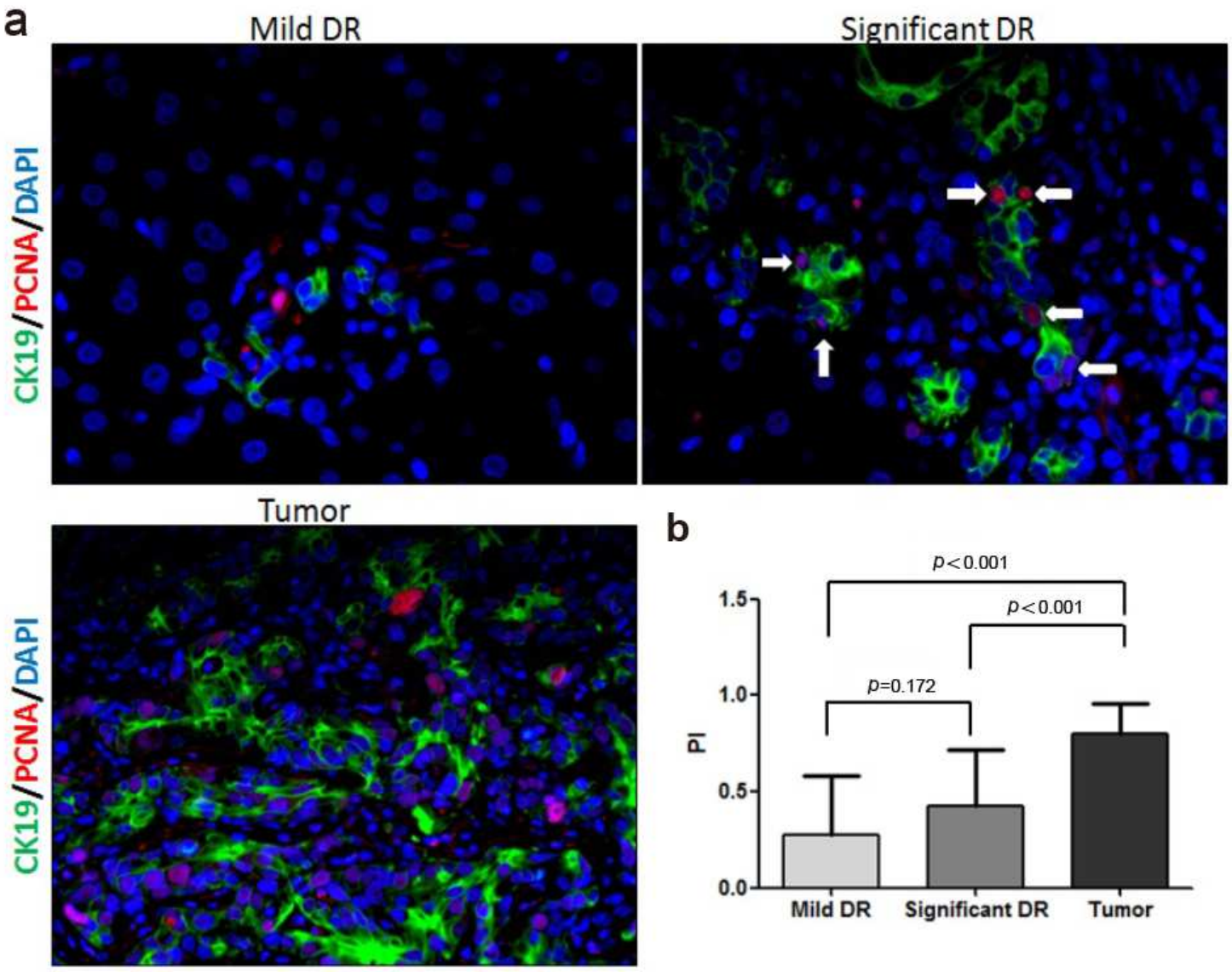

Figure 4

PI in the different peritumoral DR groups and tumor group. a CK19/PCNA positive expression in the mild and significant peritumoral DR group and tumor group. b The significant peritumoral DR group indicated a higher PI trend of ductular cells compared with the mild peritumoral DR group, and tumor group showed much higher PI compared with the other two groups. [PI = number of PCNA-positive (arrow)/total number of reactive ductular/tumor cells].

Figure 5

Microenvironment in the different peritumoral DR groups and tumor group. a The significant DR group had more abundant ECM and α-SMA-positive vessels in peritumoral areas than in the mild DR group, and

tumor group had similar microenvironment with the significant DR group. b The correlation analysis illustrated that the DR grade was positively related to the portal/septal α-SMA level.

Supplementary Files

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- Table1.pdf