Relationship between PK2 and number of Kupffer cells during the progression of liver fibrosis in patients with HBV

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Background/aim: This study aimed to investigate the potential regulatory role of prokineticin 2 (PK2) in modulation of the number and function of Kupffer cells (KCs) during the progression of liver fibrosis in patients with hepatitis B virus (HBV).

Materials and methods: We obtained liver tissue sections from 200 patients with HBV undergoing surgical resection in our hospital between January 2013 and July 2016. Of these 200 tissue sections, 150 were fibrosis tissues and 50 were hepatocellular carcinoma tissues. Immunohistochemical evaluations were performed to assess the expression levels of CD68 and PK2 in the sections. The clinical parameters of these 200 patients were also analyzed.

Results: As a potential cytokine, PK2 was commonly expressed in KCs. In addition, a close correlation between PK2 and the number of KCs during the progression of liver fibrosis in patients with HBV was found in this study.

Conclusion: PK2 is expressed in KCs and participates in the progression of liver fibrosis after HBV infection. As a potential cytokine, PK2 modulates the number of KCs during fibrosis. Thus, PK2 most likely adjusts the number of M1 cells to modulate the role of KCs in the progression of liver fibrosis after HBV infection.

Key words: Prokineticin 2, macrophage, Kupffer cells, liver fibrosis, hepatitis B virus infection

1. Introduction
Chronic hepatitis B (CHB) (defined as hepatitis B surface antigen positive for at least 6 months) is a global health issue, and the cumulative damage caused by inflammation in the liver is an important inducement in the development of cirrhosis and hepatocellular carcinoma (HCC) (1,2). Liver fibrosis is an intrinsic response to CHB infection and is described as the excess deposition of extracellular matrix (ECM) preceding the development of cirrhosis and HCC (3,4). There is evidence to show that liver fibrosis, to some extent, and even cirrhosis can be reversed (3–5).

The host immunological response plays a key role in the natural history of chronic HBV infection (6–9). Immune cells, with the capacity to exert either injury-inducing or repair-promoting effects, play a key role in the fibrotic cascade induced by CHB (6–9).

Monocytes are circulating precursors of tissue macrophages, which help orchestrate adaptive immune responses, and infiltrating monocytes have recently been found linked to the development of liver fibrosis (3,8,9).

Macrophages play a vital role in maintaining homeostasis, promoting and resolving inflammation, repairing tissue, and causing liver fibrosis progression or regression in the pathogenesis of acute or chronic liver injury (6,9–11). Both monocytes and macrophages play a dual role in modulating the level of transforming growth factor beta 1 (TGF-β1) and other cytokines and directly adjust the replenishment of the bone marrow-derived monocyte/macrophage population during the pathogenesis of liver fibrosis (7,9,11,12).

Kupffer cells (KCs) can activate hepatic stellate cells through paracrine factors such as lipid peroxides, TGF-β1, tumor necrosis factor-α (TGF-α), interleukin-6 (IL-6), platelet-derived growth factor (PDGF), gelatinase B, reactive oxygen species (ROS), and nitric oxide (NO) during the progression of fibrosis (5,7,8,11,12). The plasticity of KCs enables them to alter their phenotypes and functions in response to different microenvironmental cues in the pathogenesis of liver fibrosis, which makes them an attractive target (4,6,11).
Prokineticin 2 (PK2), also known as Bombina variegata 8 (Bv8), belongs to the prokineticins, which is a new family of secreted peptides characterized by a completely conserved N-terminal hexapeptide (13). The conserved structure endows PK2 with conserved bioactivities including angiogenesis, neurogenesis, digestive behaviors, hormone release, gastrointestinal motility, circadian rhythms, hyperalgesia, and blood cell function and development (14–20). Previous studies reported that PK2 has potent cytokine properties and can stimulate the proliferation and mobilization of monocytes and their differentiation into macrophages (13,15,20,21). It was also reported that PK2 is expressed by KCs in the liver and is likely to participate in the differentiation and modulation of KCs function in an autocrine/paracrine manner (17).

This study aimed to investigate the potential regulatory role of prokineticin 2 (PK2) in modulation of the number and function of Kupffer cells (KCs) during the progression of liver fibrosis in patients with hepatitis B virus (HBV).

2. Materials and methods

2.1. Liver tissue samples
Liver tissue samples consisting of 101 fibrosis, 49 cirrhosis, and 50 tumor tissues were obtained from 200 patients with HBV undergoing surgical resection between January 2013 and July 2016. Of these 200 patients, 182 were diagnosed with HCC, 12 with cirrhosis, and 6 with hepatolithiasis. In patients diagnosed with HCC, tumor samples were obtained from within the tumor.

The 200 patients included in this study consisted of 153 males and 47 females with an average age of 50.5 ± 13.2 years. Access to this material was reviewed and approved by the West China Hospital Sichuan University Clinical Trials and Biomedical Ethics Committee. Three histological staging systems (Ishak, METAVIR, and Knodell) were used to evaluate the stage of fibrosis. The Ishak scoring system classifies fibrosis using a 7-point scale as follows: F0, no fibrosis; F1, fibrous expansion of some portal areas, with or without short fibrous septa; F2, fibrous expansion of most portal areas, with or without short fibrous septa; F3, fibrous expansion of most portal areas with occasional portal-to-portal bridging; F4, fibrous expansion of portal areas with marked bridging (portal-to-portal as well as portal-to-central); F5, marked bridging (portal-portal and/or portal-central) with occasional nodules (incomplete cirrhosis); F6, cirrhosis, probable or definite (22). The METAVIR scoring system classifies fibrosis using a 5-point scale: F0, no fibrosis; F1, portal fibrosis without septa; F2, few septa; F3, numerous bridging septa without cirrhosis; F4, cirrhosis (23). The Knodell scoring system classifies fibrosis using a 4-point scale:F0, no fibrosis; F1, fibrous portal expansion; F3, bridging fibrosis; F4, cirrhosis (22). Cases were found of no significant fibrosis (Ishak score 0–2, n = 49), significant fibrosis (Ishak score 3–6, n = 101), no cirrhosis (Ishak score 0–4, n = 101), and cirrhosis (Ishak score 5–6, n = 49) (24).

All patients were positive for hepatitis B surface antigen (HBsAg) for more than 6 months. Exclusion criteria included the following: other hepatitis viruses (hepatitis A, C, and E viruses), autoimmune hepatitis, drug-induced hepatitis, hepatic echinococcosis, and liver fibrosis caused by other etiologies. The detailed characteristics of these patients are described in the Table.

2.2. Clinical data
Clinical information including sex, age, alanine aminotransferase (ALT) level, aspartate aminotransferase (AST) level, and platelet (PLT) count were obtained and analyzed in this study.

2.3. Immunohistochemical (IHC) staining
Paraffin embedded liver sections were deparaffinized by 3 changes of xylene for 5 min and rehydrated in 3 changes of 100% ethanol for 5 min, 2 changes of 95% for 5 min, and one change of 80% and 75% for 5 min, and then washed by 3 changes of distilled water for 3 min.

Antigen retrieval was performed at 121 °C for 2 min, incubating in a citrate buffer, pH 6.0. Sections were allowed to rest at room temperature (RT) for 20 min. Endogenous peroxidase activity was inhibited by incubation with 3% hydrogen peroxide (15 min, RT) and the nonspecific sites were blocked with PBS/BSA 3% (40 min, RT). PK2 and CD68 detection was performed by incubating sections with primary antibodies overnight at 4 °C with rabbit polyclonal anti-PK2 IgG (ab76747, dilution 1:100, Abcam) and mouse monoclonal anti-CD68 IgG1 (ZM-0060, dilution 1:50, ZSGB-BIO). Bovine serum albumin (3%) was used instead of primary antibodies for negative controls. Appropriate secondary antibodies were diluted in appropriate diluent and incubated at RT for 1 h; DAB was used for coloration (DAB Detection Kit, Gene Tech, Shanghai, China). Positive IHC signals were indicated by yellow, brown, or tan staining. Two experienced pathologists examined the integrated slides independently. Score evaluation was based on five randomly chosen fields at 400-fold magnification for each slide.

The CD68 and PK2 immunoreactivity in tissue sections was considered negative when no positive cells were stained, weak when fewer than 30% of the cells were positive, moderate when 30%–60% of the cells were positive, and strong when more than 60% of cells were stained as positive. The frequency of staining was thus evaluated as 0, 1+, 2+, and 3+ for no, weak, moderate, and strong immunoreactivity, respectively. The intensity of staining was similarly evaluated as 0, 1+, 2+, and 3+ for no, weak, medium, and strong staining, respectively. The IHC staining score was determined as the sum of the frequency and intensity scores. If the histology scores of
the two pathologists were different for the same sample, we chose the mean as final result (25).

2.4. Statistical analysis
Data analysis was performed with IBM SPSS statistics software, version 19.0 Data were analyzed using Pearson correlation analysis, one-way ANOVA, and normality tests. Results are expressed as the mean ± SD. P < 0.05 was considered statistically significant in all analyses.

3. Results
3.1. Characteristics of patients
In this study, 200 liver sections were obtained from 200 patients with HBV undergoing surgical resection between January 2013 and July 2016 in the West China Hospital of Sichuan University.

The histological stage of fibrosis was scored using Ishak criteria and 49 patients had no significant fibrosis (Ishak score 0–2), 101 patients had significant fibrosis (Ishak score 3–6), 101 patients had no cirrhosis (Ishak score 0–4), and 49 patients had cirrhosis (Ishak score 5–6).

In a comparison between the nonsignificant fibrosis group and significant fibrosis group, patients in the significant fibrosis group were older than those in the nonsignificant fibrosis group and the difference was statistically significant (P < 0.01). The significant fibrosis group had higher levels of ALT and AST than the nonsignificant fibrosis group (P < 0.05 and P < 0.01, respectively). The nonsignificant fibrosis group had a higher PLT count than the significant fibrosis group (P < 0.01). There was no difference in the sex ratio between the two groups. In a comparison between the noncirrhotic group and cirrhotic group, the noncirrhotic group had a higher PLT count (P < 0.01) and the cirrhotic group had higher levels of ALT and AST (P < 0.01). There were no differences in the sex ratio and age between the two groups. However, males were predominant in each subgroup (Table).

3.2. Detection of PK2 and CD68 in liver sections
CD68- and PK2-positive cells were large, stellate- or pyramid-shaped, and were mainly found lining sinusoidal vessels in the liver (Figure 1). PK2 was also found to be positive in monocyte/macrophage cells in inflammatory areas (Figure 2). PK2-positive cells were morphologically similar to resident macrophages (KCs). In hepatic sinusoids, PK2-positive cells were also mostly CD68-positive, but not vice versa. It is noteworthy that in this study PK2 was not always expressed in CD68-positive cells (Figure 3).

In the 200 samples, the expression level of both CD68 and PK2 varied with Ishak score (Figure 4). CD68 and PK2 shared a similar trend in expression during the progression of fibrosis. The expression of CD68 and PK2 peaked at the Ishak score of 2 and before that both CD68 and PK2 expression increased. However, after the peak, both CD68 and PK2 expressions decreased and eventually they were barely detected in sections of HCC tissues. The change in expression level of PK2 preceded that of CD68 (Figures 5 and 6).

3.3. Detection of the correlation between CD68 and PK2 in different fibrosis scoring systems
At an Ishak score of 0 or 1, no correlation between the expression levels of CD68 and PK2 was observed. However, the expression levels of CD68 and PK2 showed a correlation at Ishak scores of 2 to 6 with R(2) = 0.541, P < 0.01; R(3) = 0.442, P < 0.05; R(4) = 0.490, P < 0.01; R(5) = 0.595, P < 0.01; R(6) = 0.561, P < 0.01 (Figure 7).

At a METAVIR score of 0 or 1, no correlation between the expression levels of CD68 and PK2 was observed. The expression levels of CD68 and PK2 showed a correlation at METAVIR scores of 2 to 4 with R(2) = 0.541, P < 0.01; R(3) = 0.560, P < 0.01; R(4) = 0.561, P < 0.01 (Figure 8).

At a Knodell score of 0, no correlation between the expression levels of CD68 and PK2 was observed. The expression levels of CD68 and PK2 showed a correlation at Knodell scores of 1 to 4 with R(1) = 0.541, P < 0.01; R(2) = 0.560, P < 0.01; R(3) = 0.561, P < 0.01 (Figure 9).

Table. Characteristics of patients.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Total (n = 200)</th>
<th>Nonsignificant fibrosis: Ishak score 0–2 (n = 49)</th>
<th>Significant fibrosis: Ishak score 3–6 (n = 101)</th>
<th>No cirrhosis: Ishak score 0–4 (n = 101)</th>
<th>Cirrhosis: Ishak score 5–6 (n = 49)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>50.5 ± 13.2</td>
<td>42.8 ± 15.0</td>
<td>53.0 ± 11.4</td>
<td>49.2 ± 14.6</td>
<td>52.0 ± 10.1</td>
</tr>
<tr>
<td>Male/female</td>
<td>153/47</td>
<td>34/15</td>
<td>80/21</td>
<td>72/29</td>
<td>42/7</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>52.6 ± 83.6</td>
<td>31.6 ± 14.1</td>
<td>50.5 ± 55.4</td>
<td>34.4 ± 17.7</td>
<td>64.8 ± 74.4</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>53.5 ± 68.3</td>
<td>28.2 ± 16.4</td>
<td>47.8 ± 36.4</td>
<td>35.1 ± 26.6</td>
<td>54.4 ± 39.6</td>
</tr>
<tr>
<td>PLT (10^9/L)</td>
<td>147.9 ± 64.3</td>
<td>192.6 ± 46.3</td>
<td>150.0 ± 59.8</td>
<td>184.0 ± 52.0</td>
<td>122.6 ± 51.0</td>
</tr>
</tbody>
</table>

ALT: Alanine aminotransferase; AST: aspartate aminotransferase; PLT: platelets.
at Knodell scores of 1, 3, and 4 with \( R(1) = 0.541, P < 0.05; R(3) = 0.560, P < 0.01; R(4) = 0.561, P < 0.01 \) (Figure 9).

Thus, the expression levels of CD68 and PK2 did show a correlation in the three fibrosis scoring systems. It should be pointed out that when the expression levels changed, the change in PK2 expression tended to occur before the change in CD68 expression level.

4. Discussion

Fibrogenesis is an innate response of the liver to chronic injury, and of all common etiologies of fibrogenesis, CHB is a main etiology in China (2). With protracted damage caused by CHB infection, sustained fibrogenesis results in scar tissue formation in the liver, which generally leads to cirrhosis, resulting in mortality and morbidity (1,2).

A growing recognition that cirrhosis may be reversible renders fibrosis and cirrhosis amenable to treatment (3–5). It is expected that efficient antifibrotic therapy can rebuild the normal liver accompanied by the restoration of liver function and regression of clinical manifestations (26). Thus, efficient antifibrotic therapies are urgently needed. Unfortunately, to date, few antifibrotic agents are available (10). Antifibrotic treatment has enormous potential but also has high risks due to its relative lack of sensitivity (10,27–29).

Innate immune cells contribute to the pathogenesis of hepatic fibrosis (3,17,30). Previous studies revealed that, in agreement with the unidirectional irreversible process of liver fibrosis, hepatic macrophages had dual functions in experimental liver fibrosis (3,5,6,31).

KCs as liver-resident macrophage cells have a proinflammatory M1 phenotype and a profibrogenic M2 phenotype and function according to disease kinetics and environmental cues (4–6,11,32).

During injury, the M1 phenotype initiates the response to injury by rapidly producing proinflammatory cytokines and chemokines, including IL-1β, TNF-α, interferon-γ (IFN-γ), ROS, NO, CCL2, and CCL5 (5–8,11,32,33). In contrast to the M1 phenotype, the M2 phenotype exhibits potent antiinflammatory activity and promotes wound healing and fibrosis (4,6,32). These cells also antagonize the proinflammatory activity of the M1 phenotype by releasing IL-10, resistin-like molecule-α (RELM-α), chitinase-like protein, programmed death ligand-2 (PDL-2), and arginase I (ARG I), helping to resolve inflammation and initiate the wound-healing response.
and tissue homeostasis (6,11,34–39). M2 phenotype-derived TGFβ1, PDGF, tissue inhibitors of matrix metalloproteinases (TIMPs), and connective tissue growth factor (CTGF) activate the transformation of fibroblasts into myofibroblasts to stimulate the synthesis of ECM and induce the expression of TIMPs (6,11,34,40). On the other hand, the M2 phenotype can express mediators that induce apoptosis of myofibroblasts and serve as antigen-presenting cells (APCs) to promote antigen-specific TH2 and Treg cell responses, resulting in the promotion of wound healing while limiting the development of fibrosis (41–43).

PK2 belongs to a novel family of secreted proteins named prokineticins, which are characterized by an identical amino acid terminal sequence of AVITGA and a ten-cysteine residue sequence (14,17,44). PK2 was previously demonstrated to play an important role in angiogenesis, reproduction, carcinogenesis, and survival of neurons as well as the secretion of hypothalamic hormones, circadian rhythms, and the modulation of feeding (14–20). New evidence shows that PK2 is expressed by bone marrow cells and circulating leukocytes (21,45,46). In addition, PK2 was shown to directly promote survival, mobilization, and differentiation of granulocytic and monocytic lineages (14). PK2 is a potent chemoattractant for monocytes and macrophages and is able to stimulate the release of proinflammatory cytokines from macrophages and monocytes (15,21). In this study, PK2 was expressed in both KCs and infiltrating monocytes/macrophages. Taken together, these findings suggest that PK2 may be

Figure 3. The distribution features of CD68-positive cells and PK2-positive cells: A) distribution of CD68-positive cells; B) distribution of PK2-positive cells. CD68-positive and PK2-positive cells: black arrows; CD68-positive but PK2-negative cells: red arrows.
a component of the role that cytokines-chemokines play in fibrogenesis, which is completed by the initiation of KCs and maintained by the subsequent recruitment of monocytes/macrophages.

KCs act in a highly dynamic and complex way to maintain homeostasis in the liver (47). As the liver is constantly at risk of exposure to antigens and bacterial endotoxins, the liver’s immune system has developed many mechanisms to avoid ‘accidental’ activation (6). To achieve this, KCs assist in maintaining immunological tolerance and in establishing an antiinflammatory microenvironment (6,11,34). On the other hand, KCs can respond to liver injury rapidly and initiate the recruitment of monocytes in both acute and chronic liver injuries and the dramatic

**Figure 4.** The expression level of CD68 and PK2 in sections (400×). The CD68 and PK2 immunoreactivity in tissue sections was considered negative when no positive cells were stained, weak when fewer than 30% of the cells were positive, moderate when 30%–60% of the cells were positive, and strong when more than 60% of cells were stained as positive. The frequency of staining was thus evaluated as 0, 1+, 2+, and 3+ for no, weak, moderate, and strong immunoreactivity, respectively. The intensity of staining was similarly evaluated as 0, 1+, 2+, and 3+ for no, weak, medium, and strong staining, respectively.
expansion of the KC population due to the massive influx of monocytes participating in the pathological progression of injury (6,11,12). PK2 expression in infiltrating monocyte/macrophage cells was also observed in this study. The efficient and accurate behavior of KCs is implemented by the divergence of polarization, namely M1 and M2 (11). There are differences between M1 and M2 in the activation pathway; proinflammatory M1 is induced by lipopolysaccharide or IFN-γ, while antiinflammatory and immunosuppressive M2 is induced by IL-4 and IL-13 (6). Studies have demonstrated that PK2 can modulate the phagocytic capacity of macrophages irrespective of the maturity and activation of macrophages, and, once stimulated by PK2, both infiltrating macrophages and KCs show chemotaxis and secrete cytokines in order to orchestrate the innate immune system and respond to injury (3,5,6,11).

When considering the potential cytokine activity of PK2 and its distribution in this study, it is reasonable to assume that PK2 is related to the function of KCs, or in other words the polarization state of KCs during liver injury.

In this study, the semiquantitative analysis of both CD68 and PK2 revealed that KCs show an initial increase and a gradual decrease in PK2 expression during fibrosis progression and PK2 is barely detected in HCC, which may be related to inflammatory activity and fibrosis. We demonstrated that PK2 correlated with the number of KCs during fibrogenesis. The change in PK2 expression tended to precede the change in the number of KCs and it was confirmed in previous studies that PK2 participates in modulating the number of KCs. In this study, PK2 showed increased and then decreased expression in KCs in the later stage of fibrosis, which resulted in the inhibition of chemotaxis and infiltration of monocytes and may consequently lead to inhibition of the M1 phenotype. It follows that with an imbalance between proinflammatory M1 and antiinflammatory and profibrotic M2, the synthesis of ECM surpasses decomposition and consequently protracted fibrogenesis leads to cirrhosis.

In conclusion, we can speculate that it may be the M1 phenotype that is modulated by PK2 during liver injury. However, this speculation requires further study. There is no doubt that macrophages are flexible and can switch...
Figure 7. The correlation between CD68 and PK2 in the Ishak scoring system. IHC: Immunohistochemistry; CD68 and PK2 shared a similar trend in expression during the progression of fibrosis. There existed a correlation between CD68 and PK2 in the Ishak scoring system. *: P < 0.05, indicating that there was a significant difference.

Figure 8. The correlation between CD68 and PK2 in the METAVIR scoring system. IHC: Immunohistochemistry; CD68 and PK2 shared a similar trend in expression during the progression of fibrosis. There existed a correlation between CD68 and PK2 in the METAVIR scoring system. *: P < 0.05, indicating that there was a significant difference.

Figure 9. The correlation between CD68 and PK2 in the Knodell scoring system. IHC: Immunohistochemistry; CD68 and PK2 shared a similar trend in expression during the progression of fibrosis. There existed a correlation between CD68 and PK2 in the Knodell scoring system. *: P < 0.05, indicating that there was a significant difference.
from one functional phenotype to another in response to variable signals in the microenvironment in order to exert their pro- and antiinflammatory and pro- and antifibrotic effects. PK2, as a cytokine expressed by KCs in the liver, plays a key role in modulation of the activity of KCs. This study determined the correlation between PK2 and the number of KCs during the progression of liver fibrosis in patients with HBV based on the Ishak scoring system. However, it should be noted that the modulation of PK2 on KCs is not exclusive, as the activity of KCs can be modulated by other mechanisms. Nevertheless, it is the important effect of PK2 on the activity of KCs that provides new insights into the potential for therapeutic strategies for fibrosis. PK2 may be a new therapeutic target. A change in PK2 via exogenous drugs constitutes a promising strategy for the treatment of liver fibrosis in patients with CHB.

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References


