

ACE2 and TMPRSS2 Expression in Hepatocytes of Chronic HBV Infection Patients

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Abstract

Background: Pre-existing liver disease is a risk factor for the worse prognosis of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection. We aimed to evaluate whether chronic hepatitis B (CHB) and hepatocellular carcinoma (HCC) affect the expression of viral receptor angiotensin-converting enzyme 2 (ACE2) and transmembrane serine protease 2 (TMPRSS2) in the liver.

Methods: Twelve pairs of matched liver tissues of HCC and para-carcinoma were collected from the First Affiliated Hospital of Zhejiang University School of Medicine. And 20 liver biopsies from CHB patients were collected from Peking University People's Hospital. The expression of ACE2 and TMRPSS2 were detected using immunofluorescence staining, western blot, and RT-qPCR. The effects of hepatitis B virus (HBV) replication or interferon on ACE2 and TMPRSS2 expression were tested in hepatic cell lines.

Results: The mRNA expression of TMPRSS2 in HCC tissues was six-fold higher than that of para-carcinoma tissues ($P=0.002$), whereas that of ACE2 was not statistically different between HCC and para-carcinoma tissues. Hepatocellular ACE2 expression was detected in 35% (7/20) of CHB patients and mostly distributed in the inflammatory areas. However, there was no difference in TMPRSS2 expression between areas with or without inflammation. IFN- α 2b slightly induced ACE2 expression (2.4-fold, $P=0.033$) in HepG2 cells but not in Huh-7, QSG-7701, and L-02 cells. IFN- α 2b did not affect TMPRSS2 expression in these cell lines. In addition, HBV replication did not alter ACE2 expression in HepAD38 cells.

Conclusions: Although HBV replication does not directly affect the expression of ACE2 and TMPRSS2, intrahepatic inflammation and carcinogenesis may increase their expression in some patients, which, in turn, may facilitate SARS-CoV-2 infection in hepatocytes.

Keywords: ACE2; Liver diseases; SARS-CoV-2; TMPRSS2

Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the causative agent of COVID-19, which led to the most extensively spread and most difficult to contain pandemic since the Spanish influenza pandemic in 1918. This virus can cause respiratory infections with severity ranging from mild cold- and flu-like symptoms to fatal pneumonia. In severe cases, it can result in the dysfunction of other organs such as the kidneys and liver in a substantial proportion of patients.^[1,2] SARS-CoV-2 is dependent on angiotensin-converting enzyme 2 (ACE2) as the

receptor for entry into cells and the host transmembrane serine protease 2 (TMPRSS2) for the viral spike (S) protein priming.^[3,4] ACE2 is abundantly expressed in a variety of cells in different human organs, including lung alveolar and small intestinal epithelial cells, vascular endothelial cells, smooth muscle cells, proximal tubular cells in the kidney, and basal epidermal layer of the skin, as well as in the oral and nasal mucosa.^[5]

Clinical data revealed that 14.8%–53% of COVID-19 patients had a liver injury during either viremia or inflammatory phase. Significant liver injury with elevated levels of alanine aminotransferase (ALT), bilirubin, alkaline phosphatase, and γ -glutamyl transpeptidase has been reported in 58%–78% of patients with severe clinical manifestations, with liver injury being a surrogate marker for the adverse outcomes of COVID-19.^[2,6] In addition, pre-existing liver diseases, including chronic viral hepatitis, steatohepatitis, cirrhosis, and hepatocellular carcinoma (HCC), are independent risk factors for the severity and fatality of the disease.^[7–12] However, previous studies suggested that ACE2 might be low or absent in hepatocytes, and higher expression of ACE2 is exhibited in biliary cells than in hepatocytes.^[5,13] Thus, whether the liver injury is caused by direct viral damage, drug toxicity, hypoxia, or microthromboses remains controversial.^[2,14]

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Infectious Diseases & Immunity (2021) 1:1

Received: 24 December 2020

<http://dx.doi.org/10.1097/ID9.000000000000007>

Through ultrastructural examination, a recent study has reported that typical coronavirus particles can be found in the cytoplasm of hepatocytes of COVID-19 patients, indicating that SARS-CoV-2 is capable of infecting hepatocytes directly in some cases.^[15] In the present study, we retrospectively analyzed the expression of ACE2 and TMPRSS2 in the liver tissues of patients with pre-existing liver conditions of HCC or chronic hepatitis B (CHB). In addition, we detected potential host factors that may regulate ACE2 and TMPRSS2 expression. We aimed to determine whether these liver diseases could modulate the intrahepatic ACE2 and TMPRSS2 expression, which, in turn, may affect the susceptibility of hepatocytes to SARS-CoV-2 infection.

Methods

Ethical issues

This study was conducted following the ethical guidelines of the Declaration of Helsinki and was approved by the ethics committee of Hangzhou Normal University (2020-092). Because existing residual specimens were used in the study, the requirement to obtain informed patient consent was waived by the ethics committee.

Study subjects

Paraffin-embedded liver biopsy samples were obtained from CHB patients who were recruited for clinical studies on Peg-IFN- α 2b (Schering-Plough, New Jersey, USA) therapy in Peking University People's Hospital from October 2009 to October 2011.^[16,17] Patients who met the following criteria were included: adults (18–70-years old), positive for HBsAg for more than 6 months, positive for hepatitis B e antigen, hepatitis B virus (HBV) DNA >20,000 IU/mL, and elevated serum ALT value 2 to 10 times the upper limit of the normal range. Patients with liver diseases other than CHB, pregnant and/or breastfeeding women, individuals who had been administered with immune regulators or underwent antiviral therapy within the previous 6 months, individuals with decompensated or compensated cirrhosis, individuals with antibodies against human immunodeficiency virus, and individuals with a history of renal dialysis and/or organ transplantation were excluded.

Matched HCC tissues and para-carcinoma tissues were previously collected from patients who underwent surgery at the First Affiliated Hospital of Zhejiang University School of Medicine. The HCC was developed on the basis of liver cirrhosis and chronic HBV infection.

Cell culture

Hepatic cell lines HepG2, Huh-7, QSG-7701, and L-02 were maintained in complete DMEM (Invitrogen, Carlsbad, CA, USA). Cells were treated with 100 IU/mL of IFN- α 2b (Sangon, Shanghai, China) for 2 days and collected for analysis. The HepAD38 cell line (kindly provided by Prof. Christoph Seeger from Fox Chase Cancer Center, Philadelphia), which supports HBV replication under tetracycline control, was routinely maintained in complete DMEM supplemented with 380 μ g/mL G418 antibiotic and 1 μ g/mL tetracycline (Sigma, St. Louis, MO, USA). To support the stable production of HBV, tetracycline was removed from the culture medium at least 2 weeks prior.

RNA isolation and quantitative reverse-transcription polymerase chain reaction

Total RNA was isolated using a TRIzol reagent (Invitrogen) and used for one-step quantitative reverse-transcription polymerase chain reaction (RT-qPCR) analysis (NEB, Ipswich, MA, USA). The housekeeping gene β -actin was used as a control to normalize variations. Primers for amplifying target genes were synthesized (SBS bio, Beijing, China). The sequences of primers for ACE2 were as follows: Forward: 5'-AAACATACTGTGACCCCGCAT-3'; Reverse: 5'-CCAAGCC TCAGCATATTGAACA-3'. The sequences of primers for TMPRSS2 were as follows: Forward: 5'-ACAACCT GATCACACCAGCC-3'; Reverse: 5'-GAT-TAGCCGTCTG CCCTCAT-3'. The sequences of primers for β -actin were as follows: Forward: 5'-GC GGGAAATCGTGCGT-GACATT-3'; Reverse: 5'-GATGGAGTTGAAGGTAGTTTCG TG-3'.

Immunofluorescence microscopy

Paraffin-embedded liver biopsy samples were deparaffinized and hydrated before immunostaining. Cells were probed with primary antibodies against ACE2 (Rabbit-IgG), TMPRSS2 (Rabbit-IgG), and CK18 (Mouse-IgG) and were visualized using anti-mouse-488 and anti-rabbit-594 Alexa Fluor-conjugated secondary antibodies (Abcam, Chicago, IL, USA). As a marker of hepatocytes, CK18 was used to indicate hepatocyte distribution. Images of more than 2000 cells from ten visual fields with or without infiltration of inflammatory cells were captured for analysis. For comparability of signal strength, identical exposure times were used to capture the images.

Western blotting

Cells were lysed with 1 \times Laemmli buffer, and a fraction of the cell lysate was separated on 10% polyacrylamide gels and transferred to polyvinylidene difluoride membranes. The membranes were probed with a primary antibody for ACE2 (1:2000 dilution), followed by incubation with secondary antibodies conjugated with horseradish peroxidase. The housekeeping protein β -actin (Abcam) was used as a control. Chemiluminescence signals were detected (Sigma), and the image was digitized using QuantityOne software (Bio-rad, Hercules, CA, USA).

Statistical analysis

Statistical analysis was performed using SPSS V.16.0 software (SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered statistically significant (all tests were two-tailed). Analytical methods were selected based on data characteristics. Differences between the two groups were compared using paired Student t -tests or Mann-Whitney U test.

Results

Patient characteristics

A total of 20 CHB patients were included in this study. Baseline liver biopsy samples were used for the detection of ACE2 and TMPRSS2 expression. The mean age, serum ALT, and HBV DNA of patients were 30.5 years, 140 IU/L, and 9.65E + 8 IU/mL at baseline, respectively. A total of 12 pairs of matched HCC tissues and para-carcinoma tissues were included in the study.

Table 1: Characteristics of the study population

Characteristics	Chronic hepatitis B	Hepatocellular carcinoma
Gender		
Male	18	11
Female	2	1
Age at recruitment (years)		
≤29	11	0
30–39	7	0
40–49	2	2
50–59	0	6
60–69	0	4
ALT level		
Normal	2	7
Abnormal	18	5
HBV DNA level (copies/mL)		
<10 ⁴	0	6
10 ⁴ –10 ⁵	0	4
10 ⁵ –10 ⁶	1	2
10 ⁶ –10 ⁷	1	0
10 ⁷ –10 ⁸	4	0
>10 ⁸	14	0
HBeAg		
Positive	20	6
Negative	0	6

ALT: glutamic pyruvic transaminase; HBV: hepatitis B virus; HBeAg: Hepatitis B e antigen.

The mean age of HCC patients was 56 years. All patients were infected with HBV, and half of them had positive hepatitis B e antigen levels. The detailed characteristics of these CHB and HCC patients are described in Table 1.

TMPRSS2 expression in HCC tissues was significantly higher than that in para-carcinoma tissues

Relatively low levels of ACE2 mRNA (mean value of ACE2/Actin was $\sim 10^{-3}$ – 10^{-2}) were detected in the 12 pairs of matched liver tissues. There was no significant difference in the ACE2 mRNA levels between HCC and para-carcinoma tissues [Figure 1A and 1C, $P > 0.05$]. However, TMPRSS2 mRNA levels were approximately 60-fold higher than ACE2 mRNA levels in the liver. Furthermore, TMPRSS2 mRNA levels in HCC tissues were approximately 6-fold higher than that in para-carcinoma tissues [Figure 1B and 1D, $P = 0.002$].

ACE2 was expressed in hepatocytes and induced by inflammation in some CHB patients

CK18 is a major component of the intermediate filaments in simple epithelial cells and is often used to identify differentiated hepatocytes. As shown in Figure 2A, CK18 was expressed on the cell membrane of hepatocytes. ACE2 expression was detected on the membrane and/or cytoplasm of hepatocytes in 35% (7/20) CHB patients and was frequently detected in the inflamed areas. However, there was no significant difference in TMPRSS2 expression between areas with or without immunocyte infiltration.

To investigate whether ALT, a marker of liver inflammation, was correlated with ACE2 expression, we compared ALT levels in patients with or without ACE2 expression in hepatocytes.

However, no significant difference was detected in ALT levels between the two groups [Figure 2B].

Heterogeneity and interferon-induced ACE2 expression in the hepatic cell lines

The effect of interferon on ACE2 and TMPRSS2 expression was tested in human hepatic cell lines. The mRNA levels of ACE2 but not TMPRSS2 were mildly increased in HepG2 cells after 2 days of IFN- α 2b treatment [$P = 0.033$, Figure 3A]. However, IFN- α 2b treatment did not significantly alter ACE2 mRNA levels in QSG-7701, Huh-7, and L-02 cells (Figure 3B and 3D). Although there was a significant difference in statistics ($P = 0.001$), the change in TMPRSS2 mRNA levels was less than 2-fold (1.9-fold) in QSG-7701 cells after 2 days of IFN- α 2b treatment [Figure 3C].

Western blotting showed significant heterogeneity in ACE2 expression among the cell lines, with Huh-7 cells exhibiting the highest ACE2 expression [Figure 3E]. Consistently, IFN- α 2b treatment slightly increased ACE2 protein levels in HepG2 cells but not in other hepatic cell lines.

HBV replication did not affect ACE2 expression in HepG2 cells

To determine whether HBV replication could modulate ACE2 expression, we detected ACE2 expression in HepAD38 cells, which support HBV replication under tetracycline control. Stable HBV replication after 2 weeks of tetracycline removal did not cause a significant change in ACE2 mRNA level in HepAD38 cells, suggesting that HBV replication did not directly affect ACE2 expression [Figure 4, $P = 1.000$].

Discussion

SARS-CoV-2 can infect hepatocytes, and pre-existing liver diseases are associated with a worse prognosis in COVID-19 patients.^[2,6–12] However, whether pre-existing liver diseases increase the risk of SARS-CoV-2 infection in hepatocytes remains unclear. In this study, we revealed that HCC and inflammation can increase the expression of TMPRSS2 or ACE2 in the liver in some cases, which, in turn, may facilitate SARS-CoV-2 infection in hepatocytes and induce liver injury.

The analyses of liver tissues of HCC patients, liver biopsies of CHB patients, and hepatic cell lines showed remarkable heterogeneity in hepatic ACE2 expression among the individuals [Figures 1–3]. This indicates that hepatic infection of SARS-CoV-2 can only occur in a subset of individuals. There was no significant difference in ACE2 mRNA expression between HCC and para-carcinoma tissues. However, TMPRSS2 expression in HCC tissues was significantly higher than that in para-carcinoma tissues. In addition, TMPRSS2 mRNA expression was much higher than ACE2 mRNA expression in the liver tissue [Figure 1]. TMPRSS2 is essential for viral entry into primary target cells and viral spread in the infected host,^[8] which indicates a relatively high viral spread in the HCC tissues. However, because ACE2 expression in HCC tissues did not increase significantly, whether the increase of TMPRSS2 expression affects SARS-CoV-2 infection should be further investigated.

ACE2 protein was detectable in hepatocytes in 35% CHB patients and was mostly detected in areas with immunocyte infiltration in the liver [Figure 2]. However, our data indicated that HBV replication did not directly affect ACE2 expression [Figure 4]. These results showed that inflammatory factors, but

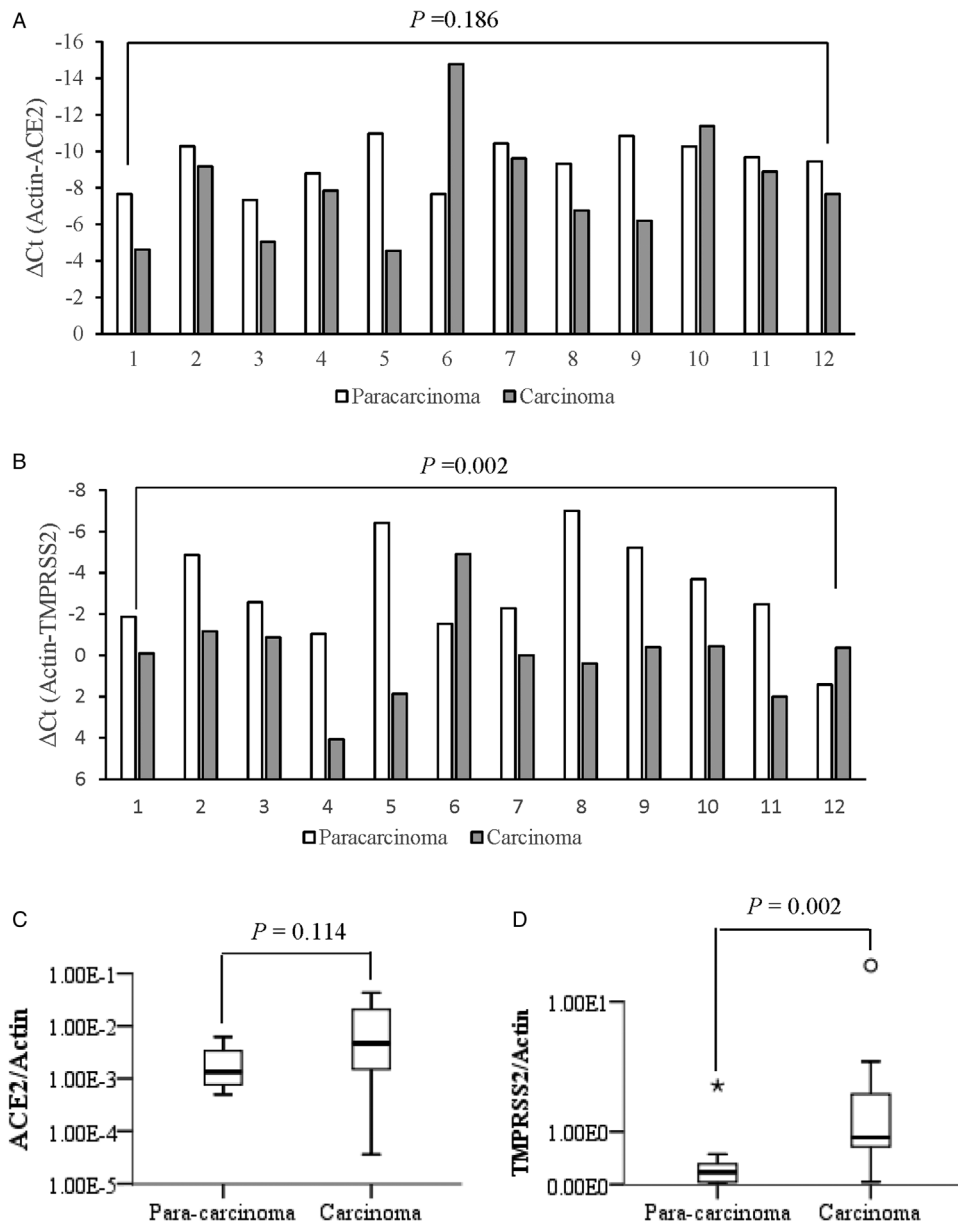


Figure 1: Intrahepatic expression of ACE2 and TMPRSS2 in patients with hepatocellular carcinoma. The levels of (A) ACE2 mRNA and (B) TMPRSS2 mRNA in 12 pairs of matched hepatocellular carcinoma and para-carcinoma tissues were analyzed using RT-qPCR and were compared using paired Student *t*-test. The value of ΔCt was the Ct value of actin minus that of target genes of ACE2 or TMPRSS2. The differences in (C) ACE2 and (D) TMPRSS2 mRNA expression between hepatocellular carcinoma and para-carcinoma tissues were compared using Mann-Whitney *U* test. $P < 0.05$ was considered statistically significant. ACE2: Angiotensin-converting enzyme 2; RT-qPCR: Quantitative reverse-transcription polymerase chain reaction; TMPRSS2: Transmembrane serine protease 2.

not viral components, can induce ACE2 expression in hepatocytes of a subset of CHB patients. However, there was no significant correlation between ALT levels and ACE2 expression [Figure 2B], which suggests that hereditary heterogeneity might be a more decisive factor than inflammation in the regulation of ACE2 expression. A previous study reported that interferon can induce ACE2 expression.^[18] In the present study, interferon only induced a slight increase in ACE2 expression in HepG2 cells but not in the other three liver-derived cell lines [Figure 3]. However, the involvement of other inflammatory factors in the induction of ACE2 expression should be further investigated. In addition, although our findings indicate that IFN enhances ACE2

expression in hepatocytes and may help in binding and entry of virions into the target cells, IFN also induces the expression of antiviral factors that can restrict viral replication. In this context, the real role of IFN-induced ACE2 expression *in vivo* remains to be further identified.

In conclusion, based on the analyses of clinical specimens, we revealed the heterogeneity of intra-hepatic ACE2 and TMPRSS2 expression and demonstrated that pre-existing liver diseases of HCC and CHB may affect the expression of TMPRSS2 and ACE2. These findings may help explain SARS-CoV-2 infection in hepatocytes in only a subset of patients and the worse prognosis of COVID-19 in patients with pre-existing liver diseases.

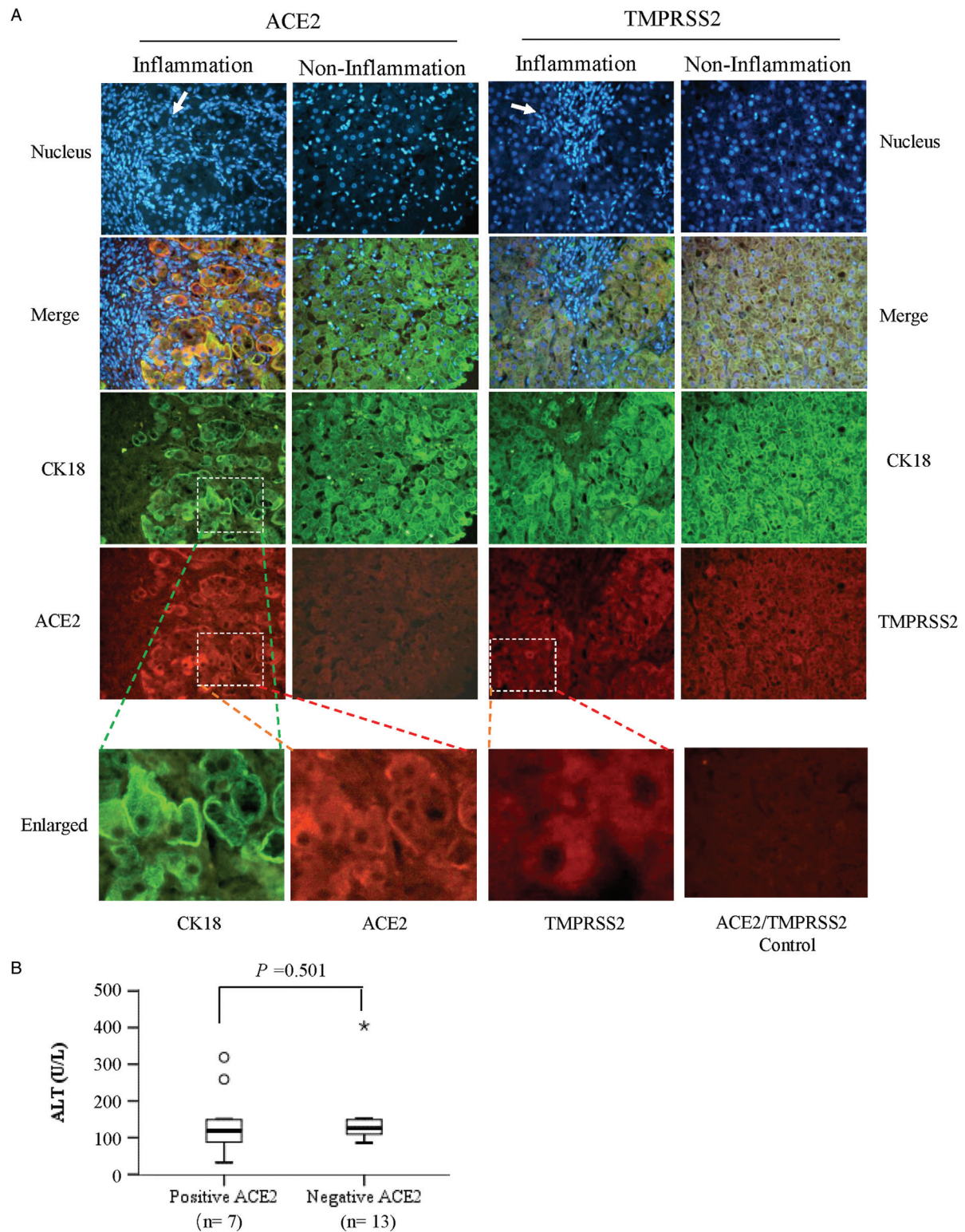


Figure 2: ACE2 and TMPRSS2 expression in the liver biopsies of CHB patients. The proteins were detected using immunofluorescence staining. CK18 was used as a marker to indicate hepatocyte distribution. The inflammatory areas were indicated with concentrated cell nuclei stained with Hoechst33342 (white arrows). These representative figures were collected from at least 10 visual fields with or without immunocytes infiltration in each slide. (A) In the controls, anti-HCV NS3 was used as the primary antibody (Rabbit-IgG). (B) The comparison of ALT levels in the CHB patients with or without ACE2 expression of hepatocyte. Student *t*-test was used to evaluate differences. $P < 0.05$ was considered statistically significant. ACE2: Angiotensin-converting enzyme 2; ALT: Alanine aminotransferase; CHB: Chronic hepatitis B; TMPRSS2: Transmembrane serine protease 2.

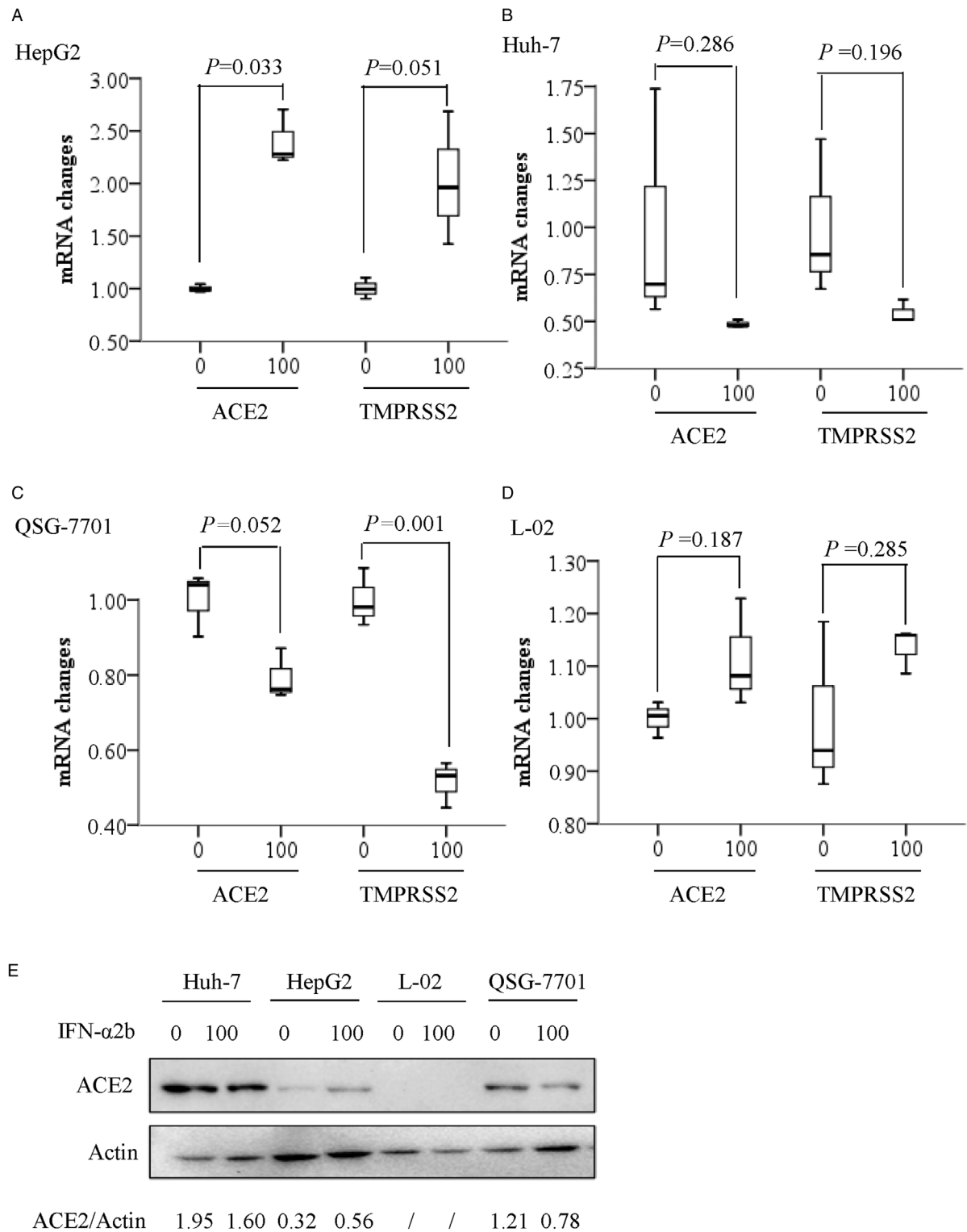


Figure 3: (A) to (D) Changes in ACE2 and TMPRSS2 mRNA of the hepatic cell lines treated with interferon. HepG2, Huh-7, QSG-7701, and L-02 cells were treated with 100IU/mL of IFN- α 2b for 2 days. The housekeeping gene β -actin was used as a control to normalize variations. (E) ACE2 protein levels were calculated based on the ratio of gray values of ACE2 to actin ($n=3$). Mann-Whitney U test was used to evaluate differences. $P < 0.05$ was considered statistically significant. ACE2: Angiotensin-converting enzyme 2; TMPRSS2: Transmembrane serine protease 2.

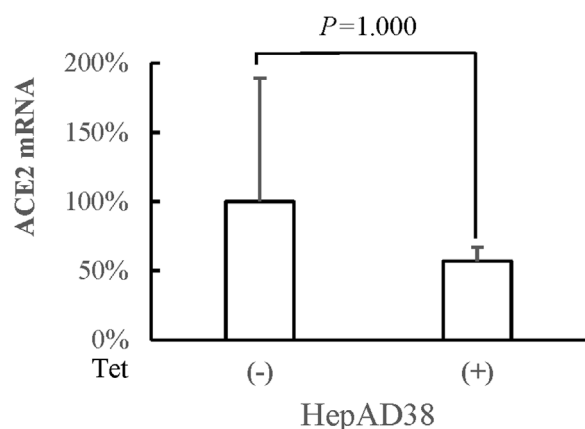


Figure 4: HBV replication does not alter ACE2 expression of HepAD38 cells. To support a stable production of HBV, tetracycline was removed from the culture medium 2 weeks prior. HepAD38 cells cultured with or without tetracycline were labeled as (+) and (-), respectively. The housekeeping gene β -actin was used as a control to normalize variations. Mann-Whitney *U* test was used to evaluate differences ($n=3$). $P < 0.05$ was considered statistically significant. ACE2: Angiotensin-converting enzyme 2; HBV: Hepatitis B virus.

Funding

This study was supported by grants from the National Natural Science Foundation of China (Nos. 82070610 and 81670530) and the start-up foundation of Hangzhou Normal University (No. 2018QDL035).

Author Contributions

Xiao-Xiao Hu and Yan-Xiu Ma performed the major part of the experiments and data analysis. Hui Ma and Sheng-Zhang Lin provided the clinical specimens. Xiang-Ji Wu, Yao-Xiang Lin and Jing Wu also performed certain experiments. Gong-Yin Chen interpreted clinical significance. Xiao-Ben Pan designed the study, interpreted the results, and wrote the manuscript.

Conflicts of Interest

None.

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Edited By Haijuan Wang

How to cite this article: Hu XX, Ma YX, Lin YX, et al. ACE2 and TMPRSS2 expression in hepatocytes of chronic HBV infection patients. *Infect Dis Immun* 2021;1(1):36–42. doi: 10.1097/ID9.0000000000000007