ATOH8 promotes HBV immune tolerance by inhibiting the pyroptotic pathway in hepatocytes

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Received January 19, 2023; Accepted April 28, 2023

DOI: 10.3892/mmr.2023.13018

Abstract. The mechanism of hepatitis B virus (HBV) immune tolerance remains unclear. Our previous studies showed that ATOH8 plays an important role in the liver tumor immune microenvironment; however, the specific immune regulatory mechanism requires further studies. Studies have shown that the hepatitis C virus (HCV) can cause hepatocyte pyroptosis; however, the relationship between HBV and pyroptosis is contested. Therefore, this study aimed to determine whether ATOH8 interfered with HBV activity through pyroptosis to further study the mechanism of ATOH8 on immune regulation and enrich our understanding of HBV-induced invasion. The expression levels of pyroptosis-related molecules (GSDMD and Caspase-1) in liver cancer tissues and peripheral blood mononuclear cells (PBMCs) of patients with HBV were assessed using qPCR and western blotting. HepG2.2.15 and Huh7 cells were used to overexpress ATOH8 using a recombinant lentiviral vector. The HBV DNA expression levels in HepG2.2.15 cells were detected using absolute quantitative (q)PCR, and the hepatitis B surface antigen expression levels in the HepG2.2.15 cell culture supernatant were measured using ELISA. The expression of pyroptosis-related molecules in Huh7 and HepG2.2.15 cells was detected using western blotting and qPCR. Additionally, the expression levels of inflammatory factors including TNF-α, INF-α, IL-18, and IL-1β were detected using qPCR and ELISA. The liver cancer tissues and PBMCs of patients with HBV showed higher expressions of pyroptosis-related molecules than those of normal samples. ATOH8-overexpressed HepG2.2.15 cells had higher HBV expression levels but lower levels of pyroptosis-related molecules, such as GSDMD and Caspase-1, than those in the control group. Similarly, the expression levels of pyroptosis-related molecules in Huh7 cells overexpressing ATOH8 were lower than that in Huh7-GFP cells. Further detection of the expression of INF-α and TNF-α in HepG2.2.15 cells overexpressing ATOH8 showed that ATOH8 overexpression increased the expression of these inflammatory factors, including those associated with pyroptosis (IL-18 and IL-1β). In conclusion, ATOH8 promoted HBV immune escape by inhibiting hepatocyte pyroptosis.

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Key words: ATOH8, hepatitis B virus, pyroptosis, immune tolerance, inflammation regulation

Introduction

Hepatitis B virus (HBV) infection is a serious condition that endangers patients worldwide. Approximately 250 million people have chronic hepatitis B (1) and are at a high risk of developing liver cirrhosis and hepatocellular carcinoma (2). Therefore, there is an urgent need for additional studies determining the mechanism of and novel treatments for HBV infection. Although current therapeutic regimens for hepatitis B are continuously being optimized, the primary treatment measures are still antiviral treatments with varying efficacy and prognostic outcomes. The limited efficacy is primarily associated with the ability of HBV to evade innate immunity through various mechanisms (3-6), including destroying the corresponding recognition receptors (7) and depleting the inflammatory factors important for the activation of adaptive immunity (8). Studies on HBV are becoming increasingly diverse, ranging from studies at the molecular and protein level, to studying the impact of the occurrence and development of viral hepatitis (9-11). However, to the best of our knowledge, only one study (12) has investigated the effects of transcription factors on the invasive activity of HBV in hepatocytes.

ATOH8, a transcription factor of the basic helix-loop-helix (bHLH) superfamily of proteins, is involved in the occurrence and development of various types of cancer, exhibiting cancer-specific effects. For example, upregulated expression of ATOH8 in the development of breast cancer is associated with a poorer prognosis (13). In contrast, in liver cancer, its overexpression plays a positive role in suppressing tumor development (14), consistent with our previous studies, which showed that overexpression of ATOH8 inhibited the migration and metastasis of liver cancer (15). However, to the best of our

knowledge, there are no studies assessing the immunoregulatory effects of ATOH8 and its role in liver cancer development.

Unlike apoptosis, pyroptosis is a form of inflammatory cell death. It involves a chain action of inflammasomes, Caspase-1, GSDMD, and other molecules that induce programmed inflammatory cell death, playing an important role in various infectious and immune diseases (16-18). Similar to the hepatitis C virus (HCV), which can induce hepatocyte pyroptosis (19); HBV can also induce several inflammatory responses; however, there are few studies on the relationship between HBV and pyroptosis.

Therefore, this study focused on the effects of ATOH8 on immune regulation in response to HBV infection. Our previous study revealed that ATOH8 enables chemotactic monocytes to secrete inflammatory factors (15). To further explore the role of ATOH8 in immune regulation, HepG2.2.15 liver cancer cells stably expressing HBV were sued to investigate whether ATOH8 interfered with the activity of HBV through pyroptosis and further explore the invasive mechanism of HBV.

Materials and methods

The experimental methods used in the present study primarily included collecting clinical samples (DNA and RNA), protein extraction, cDNA reverse transcription, qPCR, western blotting, and ELISA. In addition, a recombinant lentiviral vector was used to infect HepG2.2.15 and Huh7 cells to overexpress ATOH8. Relative RT-qPCR was used to detect the mRNA expression levels in various cell lines. Western blotting was used to detect the relative expression levels of the target proteins in the cell lines used in the present study, and ELISA was used to detect the expression of various proteins or cytokines in the cell culture supernatant. The detection of HBV expression in HepG2.2.15 cells was divided into the detection of HBV DNA in HepG2.2.15 cells (absolute RT-qPCR detection) and human hepatitis B surface antigen (HBsAg) in the culture supernatant of HepG2.2.15 cells (ELISA detection). Signed informed consent was obtained from all patients for all clinical samples used in the present study.

Clinical samples. Fresh tissue specimens of human liver cancer and adjacent non-tumor tissues were collected from patients with hepatocellular carcinoma (HCC) (9 males and 3 females, aged 35-60 years old; median age, 54.5 years) who underwent hepatectomy at the Shanghai Public Health Clinical Center, Fudan University, between March 2018 and September 2020. Blood samples were collected from patients with HBV (1 female and 3 males, aged 40-65 years old) between April 2021 and June 2022, who underwent antiviral treatment at the same hospital. Normal blood samples (2 females and 2 males, aged 30-40 years old) were collected from volunteers during the same period of time at the same hospital. The present study was approved by the Ethics Committee (approval no. 2016-S026-08) of the Shanghai Public Health Clinical Center; all patients provided written informed consent.

Cell culture. The HepG2.2.15 and Huh7 liver cancer cell lines used in the present study were all purchased from ATCC. HepG2.2.15 cells were cultured in complete MEM (BI) supplemented with 10% FBS (BI), 1% penicillin-streptomycin solution (BI) and 5 μg/ml puromycin [Yeason Biotechnology (Shanghai) Co., Ltd.] in a humidified incubator at 37˚C supplied with 5% CO_2. Huh7 cells were cultured in complete DMEM supplemented with 10% FBS, 1% PSA and 2 μg/ml puromycin. The identity of HepG2.2.15 cells used in the present study was verified by STR profiling.

Construction of ATOH8 overexpressing cells. Huh7 cells overexpressing ATOH8 that were used in the present study were constructed in a previous study (15), and the same protocol as that described in the previous study was used to construct HepG2.2.15-ATOH8 overexpressing cells, using the same pHBLV-CMVIE-ZsGreen-Puro vector. After 48 h of infection, the fluorescence status of the cells was observed under a fluorescence microscope (x10 magnification), and the cells were screened for successful infection using puromycin [Yeason Biotechnology (Shanghai) Co., Ltd.]. Validation of the successful construction of ATOH8-overexpressing cells was performed using qPCR; verification results of successful construction of Huh7-ATOH8 cells were shown in our previous study (15).

Western blotting. A total of $1x10^6$-10^7 cells were collected and lysed using RIPA lysis buffer on ice for 15-30 min and centrifuged at 4˚C, at 12,000-14,000 x g for 10 min. The supernatant was collected, 1x Loading buffer was added, and the samples were denatured by heating in a metal bath at 100˚C for 10 min and stored at -20˚C for later use. Equal quantities of protein were loaded on a 12% SDS-gel (YESEN, China), resolved using SDS-PAGE, transferred to methanol-activated PVDF membranes, blocked with 5% skimmed milk for 15-30 min, and incubated with different primary antibodies at 4˚C overnight. The following day, the membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibody for 45-60 min at 25˚C. Signals were visualized using an Immobilon Western Kit (MilliporeSigma). The antibodies used in this study were: Anti-GSDMD (1:2,000; cat. no. AF4012; Affinity Biosciences); Anti-Cleaved-Caspase-1 (1:2,000; cat. no. AF4005, Affinity Biosciences); anti-Caspase-1 (1:1,000; cat. no. ab138483; Abcam); anti-β-actin [1:2,000; cat. no. 30102ES60; Yeason Biotechnology (Shanghai) Co., Ltd.], and anti-rabbit mAb (1:4,000, cat. no. 5571S; Cell Signaling Technology, Inc.)

RT-qPCR. Total RNA was extracted using Direct-ZOL RNA Miniprep Kits, according to the manufacturer's instructions (ZYMO Research Corp.). cDNA was synthesized by reverse transcription of 1 μg RNA and qPCR was performed using a NovoStart SYNR® qPCR SuperMix Plus kit, both according to the manufacturer's protocol (Novoprotein). mRNA expression analysis was performed using Bio-Rad CFX Maestro version 4.0 (supported by the CFX-96 qPCR instrument; Bio-Rad Laboratories, Inc.). GAPDH was used as the housekeeping gene, and expression was calculated using the $2^{-\Delta\Delta Cq}$ method. The sequences of the primers used in the present study were: GAPDH forward, ACGGATTTGGTCGTATTGGG and reverse, ATCTCGCTCCTGGAAGATGG; ATOH8 forward, CAGGTGCCGTGCTACTCATA and reverse, CAGGTGCCG TGCTACTCATA; GSDMD forward, GTGTGTCAACCT

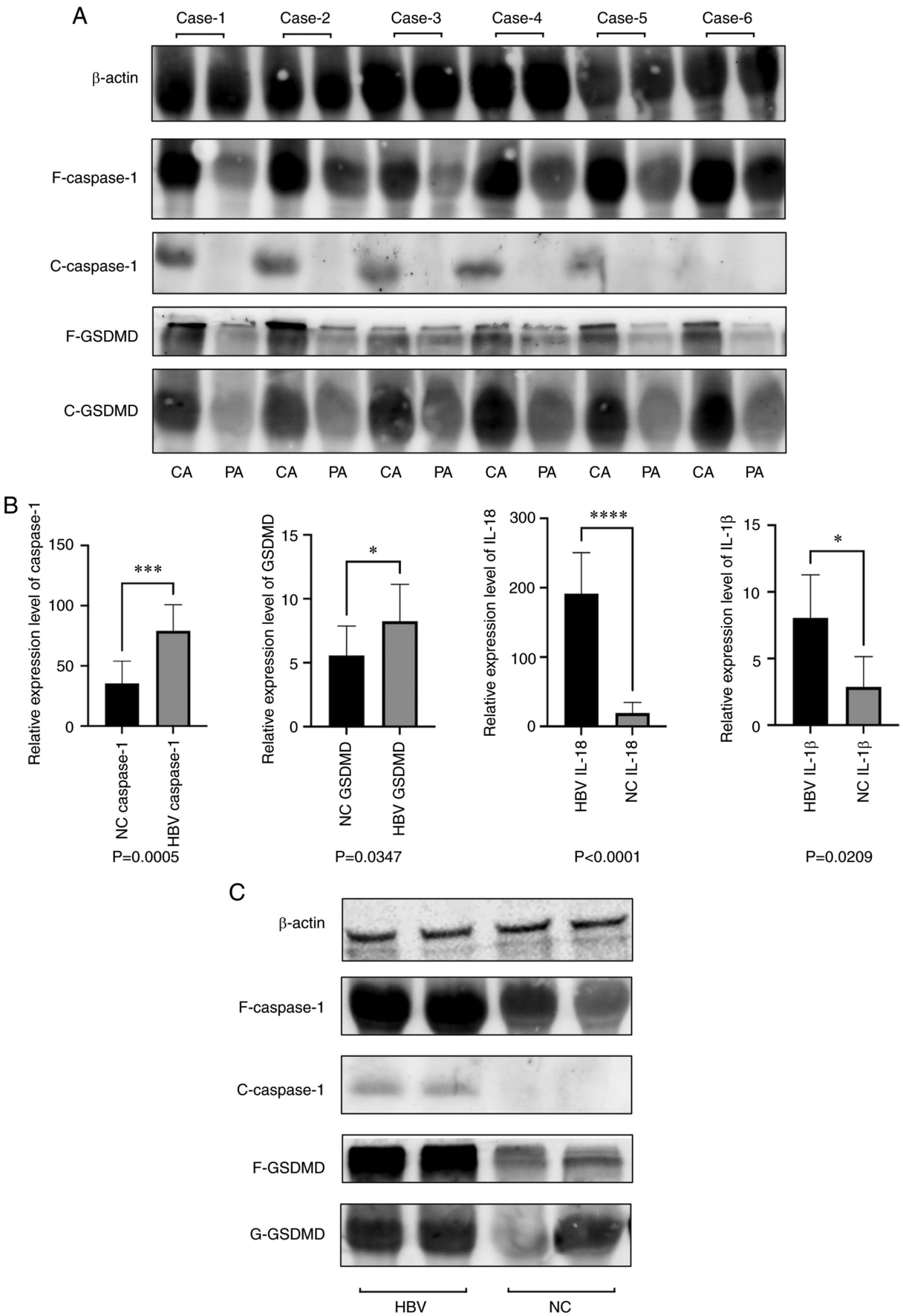

Figure 1. Expression of pyroptosis in liver cancer tissues and PBMC of HBV patients. (A) Relative protein expression levels of full-length Caspase-1, cleaved-Caspase-1, cleaved-GSDMD, and full-length GSDMD in liver cancer tissues. (B) Relative mRNA expression levels of Caspase-1, GSDMD, IL-18, and IL-1β in the PBMCs of HBV patients. (C) Relative protein expression levels of full-length Caspase-1, cleaved-Caspase-1, cleaved-GSDMD, and full-length GSDMD in the PBMCs of HBV patients. *P<0.05, ***P<0.001 and ****P<0.0001. PBMC, peripheral blood mononuclear cells; HBV, hepatitis B virus; F, full-length; C, cleaved; CA, cancerous; PA, paracancerous; NC, negative control/normal.

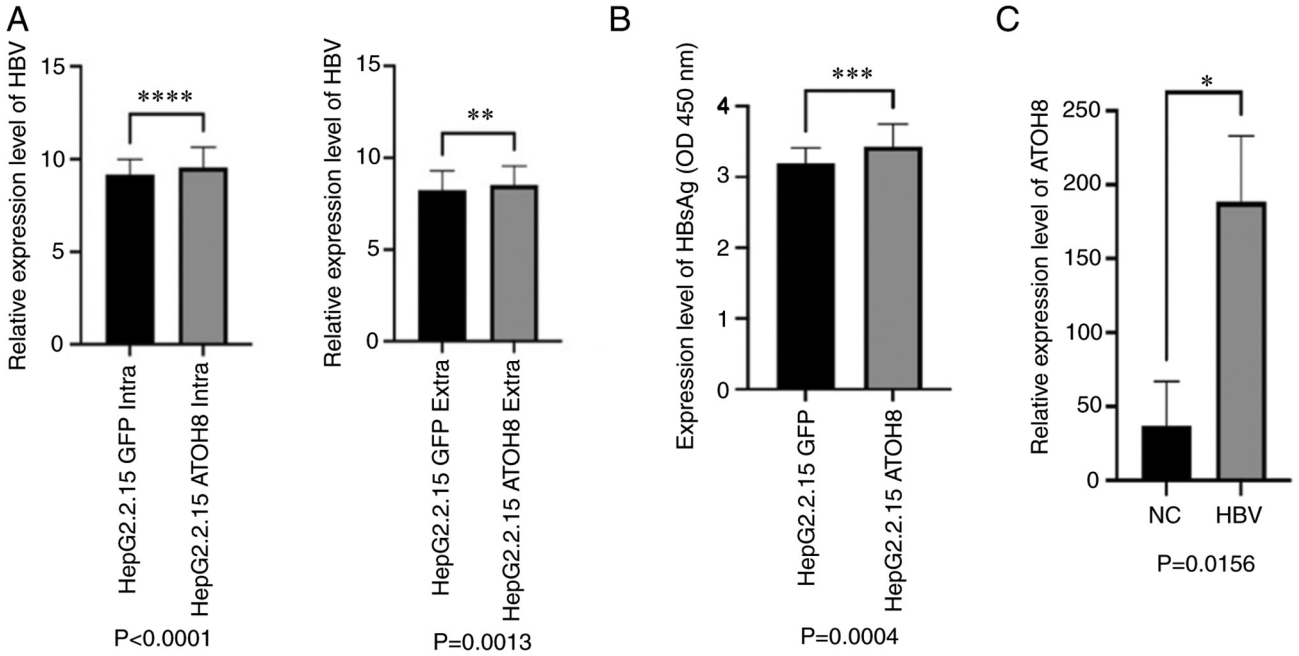

Figure 2. Effects of ATOH8 on the expression of HBV DNA in HepG2.2.15 cells. (A) Relative mRNA expression levels of HBV DNA in HepG2.2.15 cells overexpressing ATOH8. (B) HBsAg levels in HepG2.2.15 cell culture supernatant. (C) Relative expression level of ATOH8 in PBMCs of HBV patients. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001. HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; NC, negative control/normal; intra, intracellular; extra, extracellular.

GTCTATCAAGG and reverse, CATGGCATCGTAGAAGTG GAAG; Caspase-1 forward, CCTTAATATGCAAGACTC TCAAGGA and reverse, TAAGCTGGGTTGTCCTGCACT; IL-18 forward, TCTTCATTGACCAAGGAAATCGG and reverse, TCCGGGGTGCATTATCTCTAC; IL-1β forward, ATGATGGCTTATTACAGTGGCAA and reverse, GTCGGA GATTCGTAGCTGGA; TNF-α forward, TCAGGATCATCT TCTCGAACC and reverse, GAGTCCTTCTCACATTGT CTC; INF-α forward, AGAATCACTCTCTATCTGAAAGAG AAG and reverse, TCATGATTTCTGCTCTGACAACCT.

ELISA. The expression of HBsAg in the cell culture supernatant of HepG2.2.15 cells was detected using a human HBsAg ELISA kit (cat. no. JM-05282H1, JINGMEI). Absorbance (OD values) was measured at 450 nm according to the manufacturer's instructions.

HBV DNA extraction and RT-qPCR viral load analysis. The intracellular HBV DNA of HepG2.2.15 cells was extracted according to the manufacturer's instructions of the Viral DNA/RNA Kit (YESEN). The extracted DNA was directly used for qPCR (Hepatitis B Virus Nucleic Acid Assay Kit, Sansure Biotech) or stored at -20˚C (but it is recommended to proceed to the next step of qPCR immediately). qPCR was performed as above for evaluation of intracellular or extracellular HBV DNA (HBV DNA in cell culture supernatant) using the HepG2.2.15 cells. The relative HBV expression values in HepG2.2.15 cells are presented using a Log scale, as shown in Table SI.

Statistical analysis. Data were compared using a paired Student's t-tests. Densitometry analysis of the western blot bands was performed using ImageJ (version 2.3.0; National Institutes of Health). Normally distributed are presented as the mean ± SD, whereas those of skewed data are presented as the median (interquartile range). P<0.05 was considered to indicate a statistically significant difference. Data were analyzed using GraphPad Prism version 9 (GraphPad Software, Inc.).

Results

Pyroptosis is increased in liver cancer tissues and PBMCs of patients with HBV. As previously reported, the expression of ATOH8 was decreased in liver cancer (15). To detect the relationship between ATOH8 and pyroptosis, liver cancer tissues from HCC patients were assessed and it was found that pyroptosis was more common in cancerous tissues than in paracancerous tissues (Fig. 1A). To detect the relationship between HBV and pyroptosis, PBMCs were isolated from the blood samples of patients with HBV to detect the expression of pyroptotic molecules. The expression of pyroptotic molecules in the PBMCs of patients with HBV was higher than that in normal PBMCs (Fig. 1B and C).

ATOH8 increases HBV DNA expression levels in HepG2.2.15 cells. Based on our previous screening results that showed that the expression levels of ATOH8 in the HepG2.2.15 cells were very low (15), whether ATOH8 inhibited the expression of HBV, and in the process inhibited tumor proliferation and migration was assessed. The results of the establishment of the HepG2.2.15-ATOH8 cells are shown in Fig. S1. HBV DNA was extracted from HepG2.2.15 cells and qPCR was performed. Compared with the control group, HepG2.2.15 cells in the ATOH8 exhibited increased HBV DNA levels. Similar results were obtained regarding both intracellular and extracellular HBV DNA levels (Fig. 2A and B). For further verification, the cell culture supernatant of HepG2.2.15 cells was collected and the relative expression levels of HBsAg were measured using

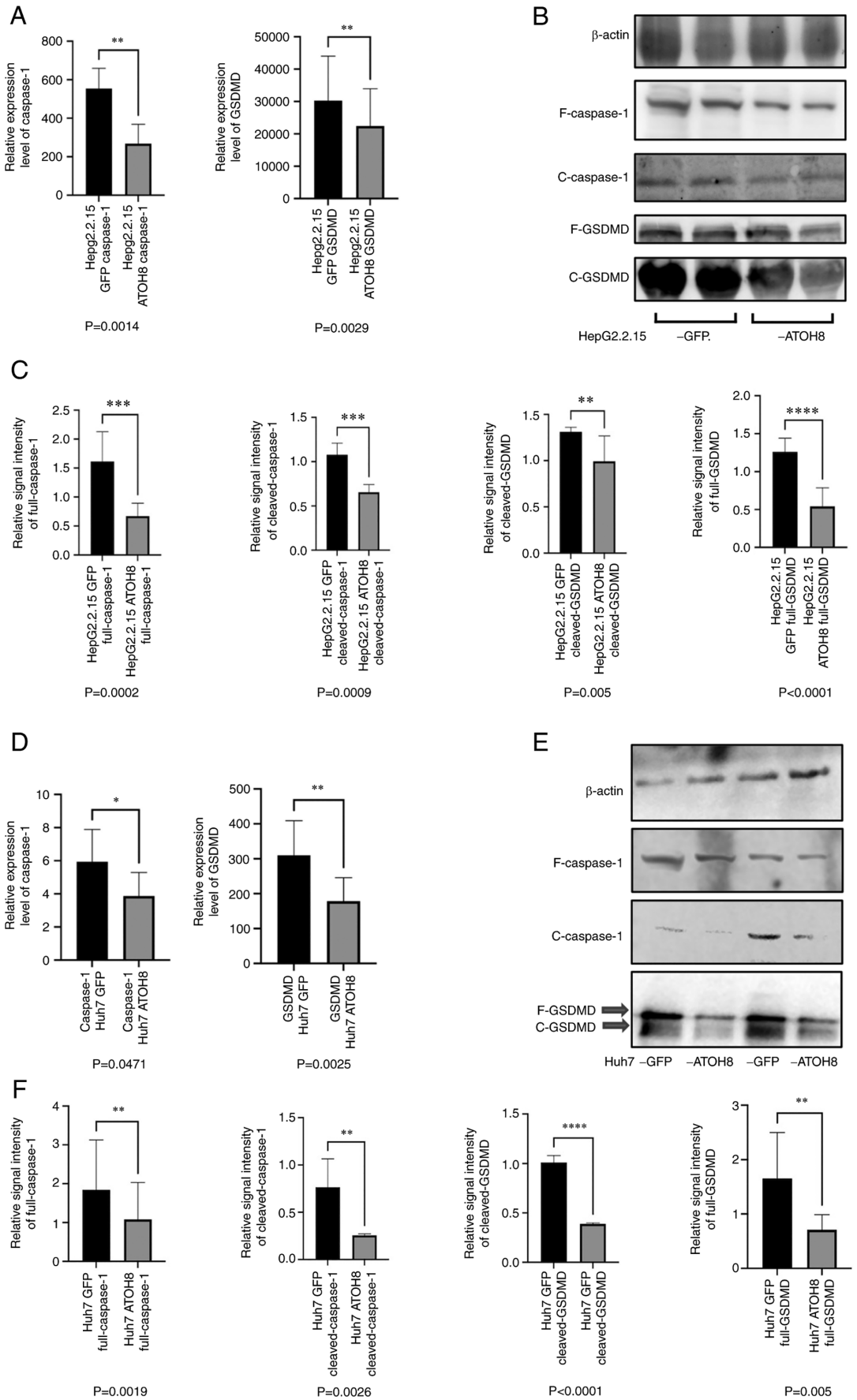

Figure 3. Effects of ATOH8 on the levels of pyroptosis-associated proteins in HepG2.2.15 and Huh7 cells. (A) Relative mRNA expression levels of GSDMD and Caspase-1 in HepG2.2.15 overexpressing ATOH8. (B and C) Relative protein expression levels of full-length Caspase-1, cleaved-Caspase-1, cleaved-GSDMD, and full-length GSDMD in HepG2.2.15 cells overexpressing ATOH8. (D) Relative mRNA expression levels of GSDMD and Caspase-1 in Huh7-GFP and Huh7-ATOH8 cells. (E and F) Relative protein expression levels of full-length Caspase-1, cleaved-Caspase-1, cleaved-GSDMD, and full-length GSDMD in Huh7-GFP and Huh7-ATOH8 cells. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001. F, full-length; C, cleaved.

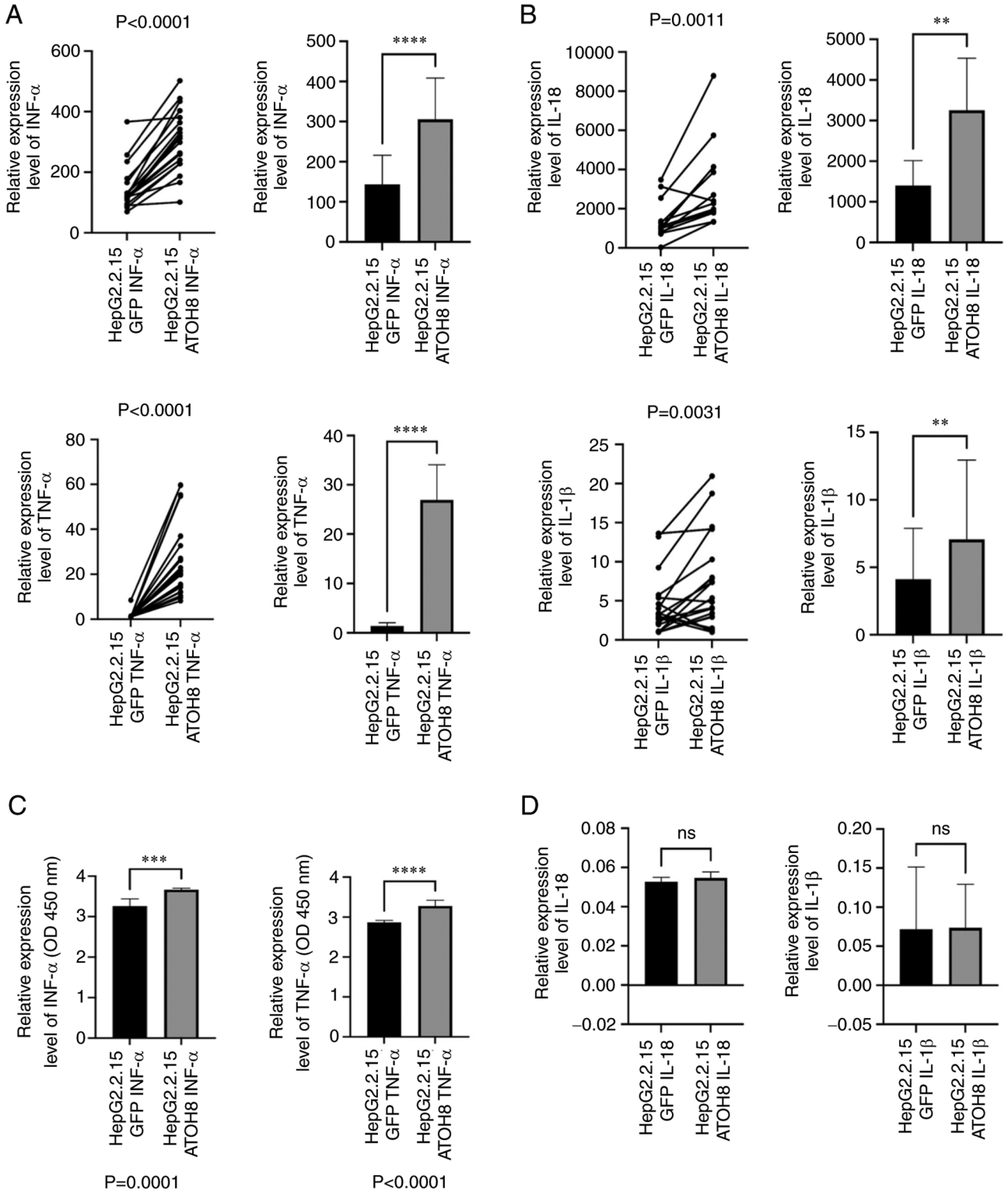

Figure 4. Effects of ATOH8 on the expression of inflammatory factors in HepG2.2.15 cells. (A and B) Relative mRNA expression levels of INF-α, TNF-α, IL-18, and IL-1β in HepG2.2.15 cells overexpressing ATOH8. (C and D) INF-α, TNF-α, IL-18, and IL-1β levels in the supernatant of HepG2.2.15 cells overexpressing ATOH8. **P<0.01, ***P<0.001 and ****P<0.0001.

ELISA and the results were consistent with those of qPCR (Fig. 2B). Similarly, the expression of ATOH8 in the PBMCs of patients with HBV was assessed; the expression of ATOH8 in patients with HBV was higher than that of normal samples, further confirming the positive association between ATOH8 and HBV (Fig. 2C).

ATOH8 inhibits the expression of pyroptosis-related molecules in HepG2.2.15 and Huh7 cells. GO analysis performed of next-generation sequencing results in Huh7 cells, performed in our previous study, showed that the activity of the NF-κB pathway regulated by AKT was inhibited in ATOH8-overexpressing Huh7 cells (15). Therefore, it was

speculated that ATOH8 may assist in HBV immune escape by inhibiting the activity of inflammatory pathways. Since NF-κB is a typical trigger pathway of pyroptosis, qPCR and western blotting were used to detect the expression of pyroptotic molecules in the HepG2.2.15 cells. Consistent results were observed between the qPCR and western blotting analyses, showing that the ATOH8 overexpression group exhibited a lower degree of pyroptosis (Fig. 3A-C). Similarly, to further verify whether ATOH8 affected hepatocyte pyroptosis, the expression of pyroptosis markers in the Huh7 cell line was detected (the hepatoma cell line with the most apparent decrease in ATOH8 expression). Compared with the ATOH8-overexpression group, the expression levels of GSDMD and Caspase-1 in the Huh7-GFP group were significantly higher (Fig. 3D-F).

ATOH8 promotes the secretion of antiviral factors in HepG2.2.15 cells. According to our previous study, ATOH8 affects the chemotaxis of monocytes and induces them to secrete inflammatory factors (15). Therefore, whether ATOH8 affected the secretion of antiviral inflammatory factors in HepG2.2.15 cells was assessed. The expression levels of INF-α and TNF-α in HepG2.2.15 using qPCR; the results were consistent with our previous study, showing INF-α and TNF-α secretion from HepG2.2.15 cells was significantly higher in the ATOH8 overexpression group than in the control group (Fig. 4A and C). Furthermore, the expression of anti-HBV inflammatory factors, such as IL18 and IL-1β, which are primarily released during pyroptosis, were assessed; and the results were consistent with the above. The expression levels of IL-18 and IL-1β in the control group were lower, but the differences were not statistically significant (Fig. 4B and D). This result contradicts the result regarding the inhibition of pyroptosis in the ATOH8 overexpression group.

Discussion

ATOH8 is a transcription factor in the basic helix-loop-helix (bHLH) superfamily that affects physiological growth and development. In the present study, the relationship between pyroptosis and HBV was assessed. It was shown that HBV escaped immune surveillance by inhibiting hepatocyte pyroptosis. Previous studies on ATOH8 have primarily focused on tumors, assessing the effect of upregulation or downregulation on tumor development. Moreover, the upregulation of ATOH8 has opposing effects in different types of cancer (13,14). However, to the best of our knowledge, only one study (20) has investigated the role of ATOH8 on inflammation.

In our previous study in which Huh7 liver carcinoma cells were used, it was shown that ATOH8 played a role in the tumor immune microenvironment. For example, ATOH8 can positively affect monocyte chemotaxis and induce the secretion of inflammatory factors (15), Therefore, to further explore the role of ATOH8 on immune regulation, HepG2.2.15 liver carcinoma cells, which endogenously express low levels of ATOH8 and stably express HBV, were used. Compared with other types of liver cancer, HBV-induced liver cancer can influence the liver microenvironment, which may be more representative of immune regulation studies (21).

It was initially hypothesized that if ATOH8 played an active role in immune regulation, there would be a decrease in the levels of HBV released from HepG2.2.15-ATOH8 cells. However, the negative results of HBV DNA in HepG2.2.15 cells and HBsAg expression in the cell culture supernatant showed higher levels of HBV in the ATOH8-overexpressing group, suggesting that ATOH8 may contribute to the immune escape of HBV. GO analysis was performed on the results of next-generation sequencing (NGS) in Huh7 cells in our previous study (15) and it was found that ATOH8 played an active role in regulating Huh7 inflammation, manifested as decreased in AKT expression and altered regulation of the NF-κB pathway activity in the Huh7-ATOH8 group. Thus, considering the important role of NF-κB during pyroptosis, it was speculated that ATOH8 assisted HBV immune escape by inhibiting HepG2.2.15 cell pyroptosis. Currently, there is only one study on the relationship between HBV and pyroptosis, to the best of our knowledge; only Yu *et al* (22) has shown that the immune tolerance of HBV was related to its action on the pyroptotic pathway, consistent with the findings of the present study. Therefore, to further verify our hypothesis, the expression of pyroptosis-related molecules in the HepG2.2.15 and Huh7 cells were determined; the results were consistent with the results of GO analysis in our previous study, showing decreased expression of pyroptosis-related molecules in the ATOH8-overexpressing cell group. Furthermore, these results were confirmed using clinical samples. Compared with the paracancerous tissues, liver cancer tissues with low ATOH8 expression showed increased expression of molecules related to pyroptosis, showing that ATOH8 could affect the expression of pyroptotic molecules.

The studies by Xie *et al* (23) and Yu *et al* (22) have results that contradict each other; the present study assessed the ATOH8 transcription factor and may explain the opposing phenomenon observed in the two studies. Xie *et al* (23) showed that HBV activates the NLRP3 inflammasome under hypoxic conditions, whereas Yu *et al* (22) showed that HBV achieved escape from immune surveillance by inhibiting the NF-κB pathway. The difference in findings may be explained by the differential expression of ATOH8 in the development of liver inflammation. During the development from normal liver cells, which rarely undergo pyroptosis, to chronic hepatitis, and then to liver cancer, expression of ATOH8 increases as the cancer develops. Following ATOH8 inhibition, HBV immune escape was reduced. Therefore, the difference between the results of the present study and those of previous studies may be due to the study of different stages of hepatitis B, and the varying roles of ATOH8 in each stage. Regarding the lower inflammasome expression levels in HBV-induced liver cancer than that in the adjacent tissues (23,24), this may be explained by the fact that following hepatocarcinogenesis, pyroptosis in liver cells is inhibited to maintain the survival state of cancer cells. In contrast, the adjacent tissues maintain a high degree of inflammatory response under HBV stimulation. This explanation does not contradict the results of the present study, in that the incidence of pyroptosis in liver cancer tissues was higher than that in adjacent tissues. Although pyroptosis is inhibited in cancer cells compared to normal tissues, cancer antigens in cancer tissues still stimulate cancer cells to induce inflammatory responses; such inflammation may be more evident without the inhibitory effect of ATOH8.

In the present study, in addition to assessing the effect of ATOH8 on HBV immune escape, the expression levels of certain antiviral factors, such as TNF-α, INF-α, IL-18, and IL-1β in HepG2.2.15 cells were also determined. Notably, the expression of these antiviral factors was higher in the ATOH8 overexpressing cells compared with the control group. In addition, the expression of certain inflammatory factors (IL-18 and IL-1β) was higher in the ATOH8 overexpressing cells, and these were likely primarily produced during pyroptosis. However, only differences in the mRNA levels of IL-18 and IL-1β were detected, and these are associated with pyroptosis primarily due to their activation by Caspase-1 (25,26). There was no significant difference in IL-18 and IL-1β with regard to the extracellular expression levels detected using ELISA between the two groups as they required cleavage of Caspase-1, and this process was blocked by ATOH8. Therefore, the expression of these antiviral factors may be a compensatory increase in response to HBV invasion, indirectly aggravating the inflammatory state during HBV invasion.

To further understand the primary antiviral mechanism of normal hepatocytes during HBV invasion, the expression of antiviral inflammatory factors and pyroptosis-related molecules in PBMCs of patients with HBV was detected. The results showed that PBMCs from patients with HBV exhibited higher expression levels of pyroptotic molecules and increased secretion of antiviral inflammatory factors. However, the expression of ATOH8 in PBMCs of patients with HBV was higher than that in normal samples, indicating that ATOH8 did not inhibit pyroptosis in PBMCs, only in hepatocytes. Therefore, considering the positive role of pyrolytic inflammatory molecules in anti-HBV activity, it is hypothesized that when HBV invades, ATOH8 inhibits hepatocyte pyroptosis, the PBMCs of patients exert antiviral effects, and the antiviral effects of hepatocytes are limited.

The present study has some limitations. For example, the findings of the present study were not validated in animal experiments. Additionally, the specific mechanism by which ATOH8 suppressed pyroptosis requires further elucidation.

In conclusion, ATOH8 interfered with the host's innate immune system by inhibiting hepatocyte pyroptosis and assisting HBV immune escape. The results of the present study may provide novel avenues for research on the mechanisms of hepatitis caused by HBV and for the development of specific immune inducers for the treatment of hepatitis B. For example, creating an inflammatory environment conducive to the expression of ATOH8 to further interfere with the progression of hepatitis B and assist in treating patients with hepatitis B with antiviral drugs.

Acknowledgements

Not applicable.

Funding

This study was supported by funding from the Shanghai Public Health Clinical Center Fund Project (grant no. KY-GW-2021-31) and the National Science and Technology Major Project (grant no. 2017ZX10203202-003-007).

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

XL and LC designed the study. JC helped designed the study. XL, ZF, and JY performed the experiments. XL and ZF analyzed the data. XL and JC wrote the manuscript. XL and JC confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The experimental protocol used in the present study conformed to the guidelines described in the Declaration of Helsinki, and was approved by the Human Ethics Committee of the Shanghai Public Health Clinical Center (approval no. 2016-S026-08).

Patient consent for publication

All patients included in the present study provided signed informed consent and agreed to the publication of their data.

Competing interests

The authors declare that they have no competing interests.

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