Original Article
ATOH8 overexpression inhibits the tumor progression and monocyte chemotaxis in hepatocellular carcinoma

Liping Chen[1#], Jingmao Yang[1#], Yajie Wang[1], Nana Wu[1], Xian Li[1], Jun Li[1], Yangqing Huang[2*], Jilin Cheng[1*]

Departments of [1]Gastroenterology, [2]Liver Surgery, Shanghai Public Health Clinical Center, Fudan University, Shanghai, China. #Equal contributors. *Equal contributors.

Abstract: Objective: ATOH8 is reported to be associated with the progression of many tumors; however, there are remaining controversies. The aim of this study is to explore the role of ATOH8 in hepatocellular carcinoma (HCC) and its effect on monocyte chemotaxis. Methods: Bioinformatics analysis was performed based on the LIHC data in GEPIA and LinkedOmic. Fresh human liver cancer and adjacent nontumor tissue specimens were collected at the Shanghai Public Health Clinical Center. qRT-PCR was performed to determine the transcript level, and western blot analysis and ELISA were used to detect protein expression. CCK8, colony formation, wound-healing, Transwell migration and invasion assays were performed to examine cell proliferation, migration and invasion. An HCC xenograft mouse model was used to determine oncogenicity in vivo. Cell apoptosis and related markers were detected by flow cytometry. Additionally, chemotaxis was assessed by the Transwell migration assay. Results: ATOH8 expression is downregulated in HCC tissue and hepatoma cell lines. High expression of ATOH8 predicts a favorable prognosis. Overexpression of ATOH8 in liver cancer cells inhibits proliferation, migration and invasion in vitro, and tumor progression in nude mice. Knockdown of ATOH8 promotes proliferation of Huh7 and EMT-related proteins. Overexpression of ATOH8 increases chemosensitivity to 5-FU, which is probably caused by inhibiting the phosphorylation of AKT (Ser473). Furthermore, overexpression of ATOH8 in Huh7 reduced MCP1 to inhibit chemotactic THP-1, and promoted antitumor inflammatory cytokine (TNF-α and IFN-γ) secretion in monocytes. Conclusion: In addition to the intrinsic oncosuppressive function of ATOH8 in the liver, ATOH8 may modulate the microenvironment to create an immune activation state. This may partly be attributed to ATOH8 inhibition of the monocyte recruitment via suppressing MCP1 expression so as to promote antitumor inflammatory cytokine secretion in monocytes.

Keywords: Hepatocellular carcinoma, ATOH8, tumor immune microenvironment, monocyte

Introduction

Liver cancer is currently one of the most commonly diagnosed cancers in the world, especially in Eastern Asia [1]. Hepatocellular carcinoma (HCC) accounts for the majority of liver cancer and ranks the fourth leading cause of cancer-related death worldwide [2, 3]. Despite a variety of advanced therapeutic strategies, the prognosis of HCC patients is still poor due to a high incidence of metastasis and recurrence [4]. Notably, HCC is representative of immunogenic tumors closely related to chronic inflammation that results in a unique tumor microenvironment (TME) [5], which includes the immune cell subpopulations and inflammatory cytokines. The immunosuppressive cells in

TME play a considerable important part in the development of HCC [6].

A number of basic helix-loop-helix (bHLH) factors are inducers of EMT and could inhibit the expression of E-cadherin [7]. Atonal homolog 8 (ATOH8), a transcription factor from the bHLH family, is reported to be associated with the progression of many tumors; however, there are remaining controversies.

For instance, ATOH8 knockdown inhibits the proliferation of colon cancer cells [8]. However, downregulation of ATOH8 contributes to the development of nasopharyngeal carcinoma [9]. The exact role of ATOH8 in HCC is still unclear. What's more, whether ATOH8 could reshape the immune microenvironment to act on HCC is

still inconclusive. The aim of this study was to further delineate the role of ATOH8 in hepatic cancer cells and its effect on the chemotaxis of monocytes.

Method and materials

Data source and processing

ATOH8 mRNA expression levels in HCC tumors were determined by analyzing the data of LIHC through GEPIA [10] by Matching TCGA tumor data and TCGA/GTEx normal data. The effect of ATOH8 on the survival of HCC patients was assessed by LinkedOmics [11].

Clinical specimens

Fresh human liver cancer and adjacent non-tumor tissue specimens were collected from 12 HCC patients undergoing hepatectomy in Shanghai Public Health Clinical Center, Fudan University. This study was approved by the Ethics Committee (2019-S047-02) of Shanghai Public Health Clinical Center and all patients signed the informed consent.

RNA extraction and qRT-PCR

Total RNA from HCC cell lines and clinical samples was extracted using (Direct-zol RNA Miniprep Kits, ZYMO, USA) according to the manufacturer's instructions. 1 ug total RNA was used for cDNA synthesize and then detected by qPCR (ChamQ Universal SYBR qPCR Master Mix, Vazyme, Nanjing, China). ABI SDS v2.3 software (Applied Biosystems) was used to analyze the relative expression level of mRNA. $2^{-\Delta\Delta CT}$ method was used to calculate the relative expression to GAPDH housekeeping control.

Construction of ATOH8 overexpression cells

Full-length ATOH8 gene (human) was amplified by PCR and then, the amplification products were cloned into the pHBLV-CMVIE-ZsGreen-Puro vector (Hanbio Biotechnology, China). Vector plasmid was shown in Figure S1A. Package Lentivirus with pSPAX2, pMD2G and vectors in 297T cells. Huh7 cells were infected with the harvest-filtered viral supernatant and selected by 2 ug/ml puromycin (Yuanpei, China). Control cells were transfected with the empty vec-

tor with GFP. Overexpression of ATOH8 was verified by qPCR and Western blot (Figure S1).

Silencing ATOH8 gene by small interfering RNA (siRNA)

According to the manufacturer's instructions, the small interfering RNA was added. Briefly, Huh7 cells were seeded in a six-well plate (2*10^5 cells/well) and cultured in DMEM without FBS for 12 h before transfection. 3 ug SiRNA and 300 ul DMEM media were mixed, and then add 6 ul the transfection reagents (Thermo, Turbofect). The mixture was incubated 25-30 minutes at 25°C and then dropped into the media. The cells were harvested for the subsequent testing purpose after 48 h.

CCK8

The cell count kit8 (CCK8, Dojindo, Japan) was performed to examine cell proliferation. 2000+ cells per well in 96-well plates were cultured for certain time. Cells incubated with CCK8 were examined under the indicated conditions after 1-2 hour(s). The CCK8-derived formazan developed by cells was measured. Finally, the OD value at 450 nm from each well was detected by using a microplate reader (EON, BioTek, USA).

Transwell/Transwell invasion assay

For Transwell assay, we used 24-well Corning Transwell Chambers (8 um, Corning Costar, Cambridge, MA, USA) according to the manufacturer's instructions. A Matrigel-precoated invasion chamber (8 um, Corning Costar, Cambridge, MA, USA) was used to detect the cell invasion.

Briefly, starved HCC cells (10^5 cells/well) were cultured in DMEM without FBS in the top chamber. The lower chamber contained 500 ul culture medium with 10% FBS was used as a chemoattractant. After 24-48 h of incubation in a 37°C incubator, the cells would migrate/invade through the lower membrane and the rest of cells would stay in the upper chamber. The cells on the upper surface of the top chamber were removed with a cotton swab. The cells which went through the basement membrane layer were fixed for 30 min using 4% formaldehyde and stained for 20 min with crystal violet (Sigma) (0.1%).

Flow cytometry

Cells were harvested and then stained with corresponding antibody according to the manufacturer's instructions (Cytofix/Cytoperm Soln Kit, BD, Becton, USA). Cell apoptosis assay (Apoptosis Detection Kit, BD, Becton, USA) was conducted to detect the cells cultured with/without 5 FU. The labeled cells were detected by flow cytometer (BD LSR Fortessa) and analyzed using FlowJo X software.

Protein extraction and western blot

According to the manufacturer's instructions, the same amount of protein (50 μg) from cells were separated in 10% SDS-PAGE and then transferred to a NC membrane; the target proteins were detected with different antibodies (4°C overnight). After washing off the primary antibodies, the membrane was incubated with HRP-conjugated secondary antibody for 1 h at room temperature. Immobilon Western Kit (MILLIPORE, USA) was used to develop the immunoreactive bands. The following antibodies were used: E-Cadherin (24E10) Rabbit mAb (1:1000, CST), N-Cadherin (13A9) Mouse mAb (1:1000, CST), Rabbit Anti-ATOH8 antibody (1:1000, ab106377, abcam), Akt (Ser473) antibody (1:1000, CST), β-Actin (13E5) Rabbit mAb (1:1000, CST), HRP labeled Goat anti-Mouse IgG (H+L) (1:10000, Pufei, China) and HRP-conjugated Goat anti-rabbit IgG (H+L) (1:10000, Pufei, China).

Tumor formation in BALB/c nude mice

To determine the ability of tumorigenic ability in vivo, we inject 1*10^7 cells of Huh7-CON cells and Huh7-ATOH8 cells subcutaneously into the right side of the hips in male BALB/c nude mice (6 w, *n*=5/group), respectively. The volume of tumors was measured twice a week. The mice were euthanized at the 20th day after transplantation, and the xenografts were removed and photographed. All animal were kept under a pathogen-free condition and the experiments were performed in accordance with the Animal Ethics Committee of Shanghai Public Health Clinical Center (2019-A042-02).

Chemotaxis assay

Monocytes (10^5 cells/ml) were seeded into the upper chamber of Transwell inserts (24-well, Corning Costar) with 300 ul 1640 without FBS. The conditional medium with 10% FBS (cultural supernatant from Huh7-GFP and Huh7-ATOH8) as respectively added into the lower chambers. The co-cultured monocytes were cultivated for 6-12 h and the migrated cells (the lower chamber) were collected.

Enzyme linked immunosorbent assay (ELISA)

The MCP-1 protein concentrations of cell culture supernate were detected with the MCP-1 Simple Step ELISA Kit (human) according to the manufacturer instructions (ab179886, abcam).

Statistical analysis

Experimental data of normal distributions were expressed as mean ± SD and independent/paired sample T test method was used to determine the difference between the two groups. The abnormally distributed measurements compared by Mann-Whitney Test. Kolmogorov-Smirnov test was used for the statistical analysis of normal distribution. $P<0.05$ was determined as statistically significant differences. GraphPad Prism 8 software was used for all the above analyses.

Results

ATOH8 is expressed at lower levels in liver cancer cell lines and hepatocellular carcinoma tissue

A total of 369 HCC cases and 160 normal control cases were collected from TCGA and GTEx database. ATOH8 expression was decreased in the HCC group compared with the control group. ATOH8 in HCC patients was decreased 2-fold in tumor versus normal tissue (P<0.05) (**Figure 1A**). An OS curve was generated by LinkedOmics [16]. HCC patients with lower ATOH8 levels had significantly shorter overall survival times than patients with normal tissue levels (N=336, P=0.01) (**Figure 1B**). The expression of ATOH8 in 12 paired HCC tissues and adjacent nontumor tissues from HCC patients was confirmed by qRT-PCR. The relative expression levels of ATOH8 (log2) were compared by the paired T test. The results shown in **Figure 1C** indicated that ATOH8 was downregulated in HCC (5.638±4.181 vs 15.32±10.85, P=0.013). ATOH8 was highly expressed in LO2 (the immortalized liver cell line) and Lx-2 (hepatic stel-
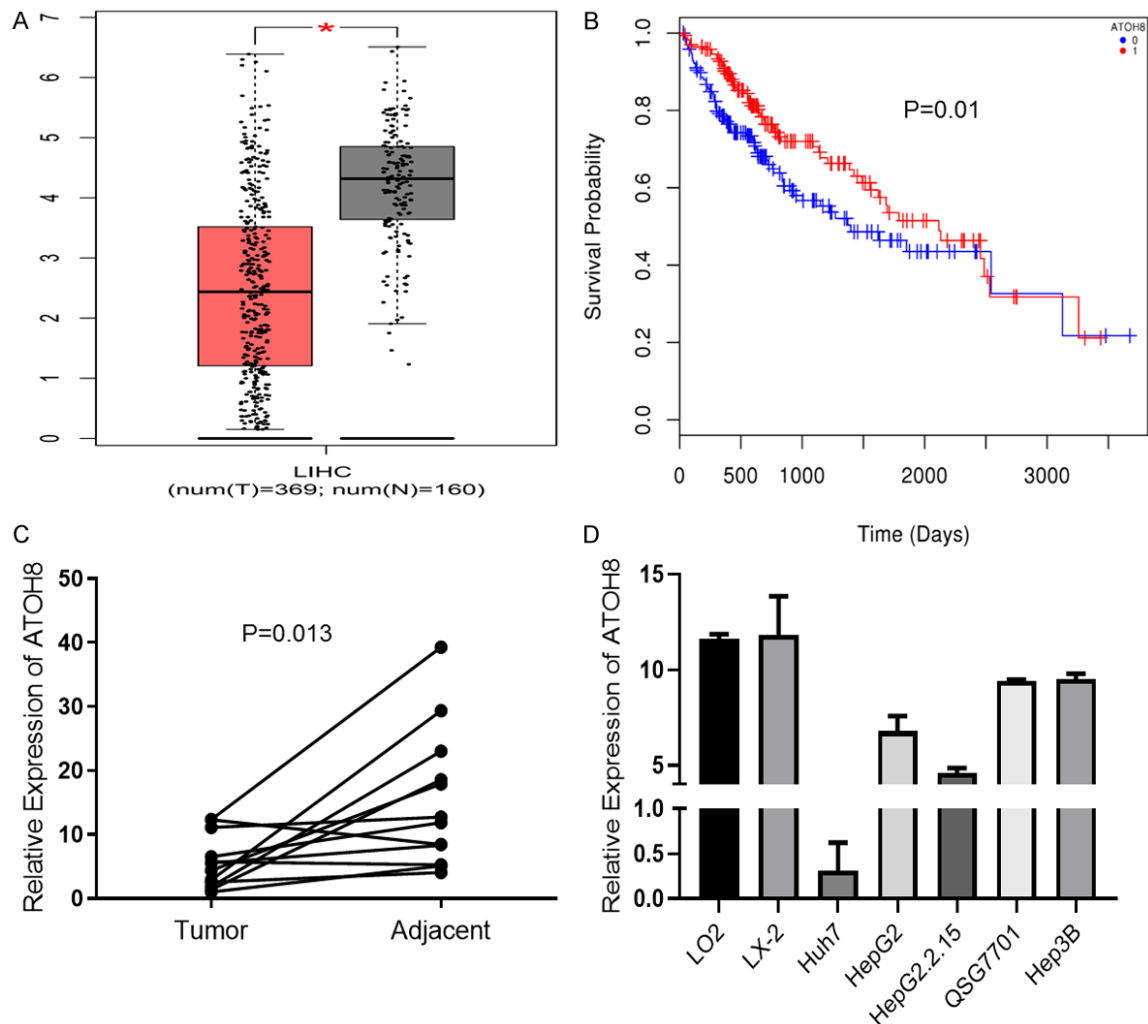
Figure 1. The expression of ATOH8 is downregulated in HCC and indicates poor prognosis. A. ATOH8 is downregulated in HCC. B. Overall survival was compared between HCC patients with high ATOH8 expression and low ATOH8 expression. C. Quantitative RT-PCR analysis of the relative expression levels of ATOH8 in 13 pairs of specimens. D. The relative differential expression of ATOH8 in several cell lines.

late cell line), while in other HCC cell lines, including QSG7701, Huh7, HepG2, HepG2.2.15 and Hep3B, its expression was relatively low (**Figure 1B**).

ATOH8 overexpression suppresses HCC cell proliferation, migration, invasion and tumorigenesis

We performed CCK8 assays to explore the effect of ATOH8 expression on the proliferation of Huh7 cells. **Figure 2A** showed the proliferation of Huh7-ATOH8 cells (overexpression) was markedly decreased compared with that of Huh7-GFP cells (control). Moreover, Transwell

and Matrigel Transwell assays demonstrated that both the migration and invasion capacities of Huh7 cells were significantly inhibited by ATOH8 overexpression (**Figure 2B**). To further evaluate the effect of ATOH8 on the growth of HCC cells in vivo, Huh7 cells (control and ATOH8 overexpression) were subcutaneously injected into nude mice. Overexpression of ATOH8 suppressed the growth of Huh7 cell xenografts in nude mice. The tumor formation rate was higher (4/5 vs 3/5), and tumor growth was faster in the control group than in the ATOH8 overexpression group (**Figure 2C**, **2D**). To further verify the function of ATOH8, we also determined the proliferation of Huh7 cells by transfection with
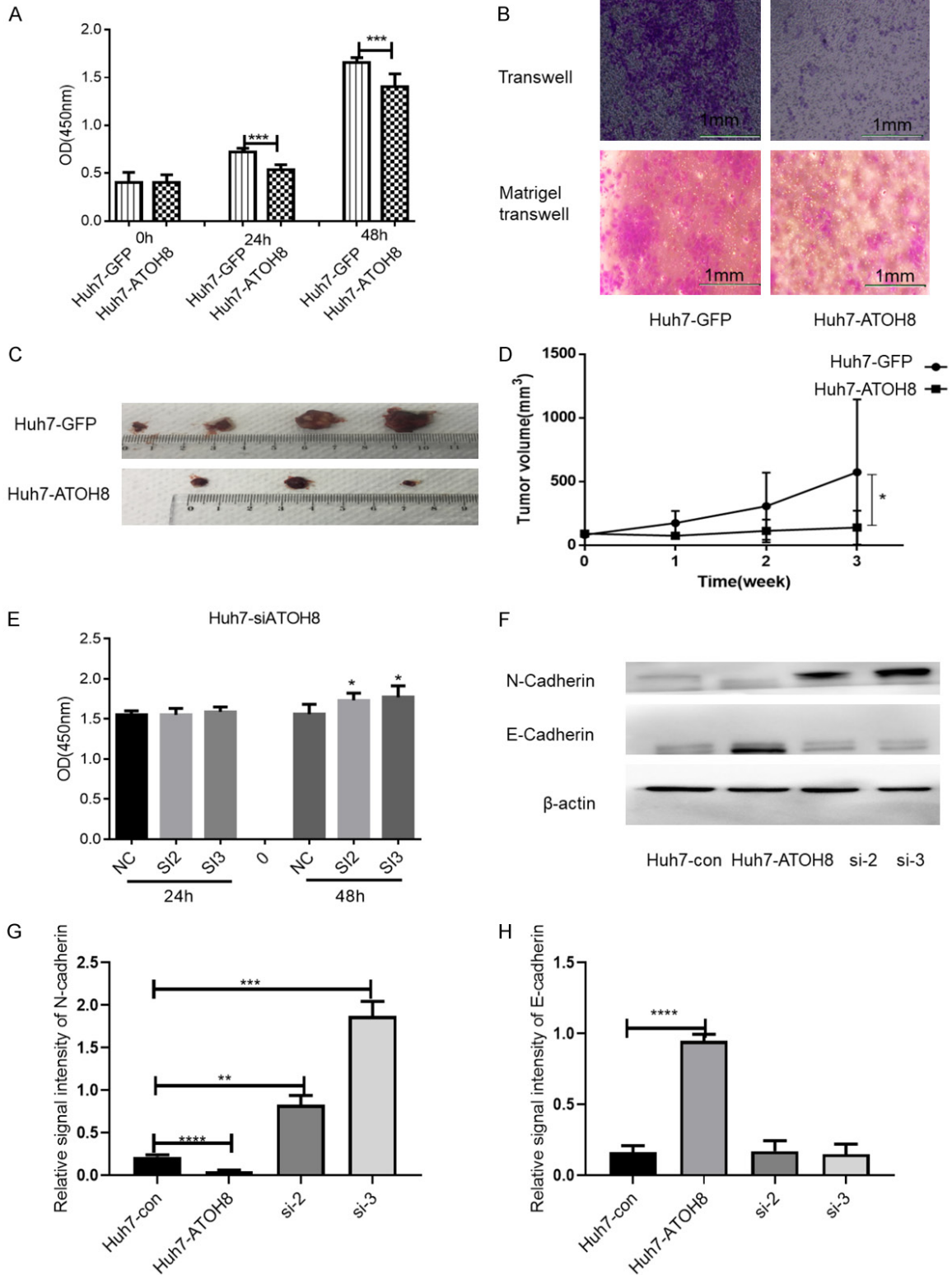
Figure 2. ATOH8 overexpression suppresses HCC cell proliferation, migration, invasion and tumorigenesis. A. The viability of Huh7 cells over time was determined by using a CCK8 assay. B. Representative images of Transwell membranes and Matrigel Transwell membranes (The scale bar was shown in the images under a quadruple objective). C. Tumors derived from nude mice induced by Huh7-ATOH8 and Huh7-GFP (control) cells (5 mice per group). D. Growth curves of tumors derived from the indicated cell lines over 3 weeks are shown. E. CCK8 assay was used to determine the cell proliferative potential after interfering with ATOH8. F. The relative expression levels of EMT-

related proteins were determined (The original lot images were shown in supplementary files). G. The relative signal intensities of N-cadherin were analyzed by ImageJ Launcher. H. The relative signal intensities of E-cadherin were analyzed by ImageJ Launcher.
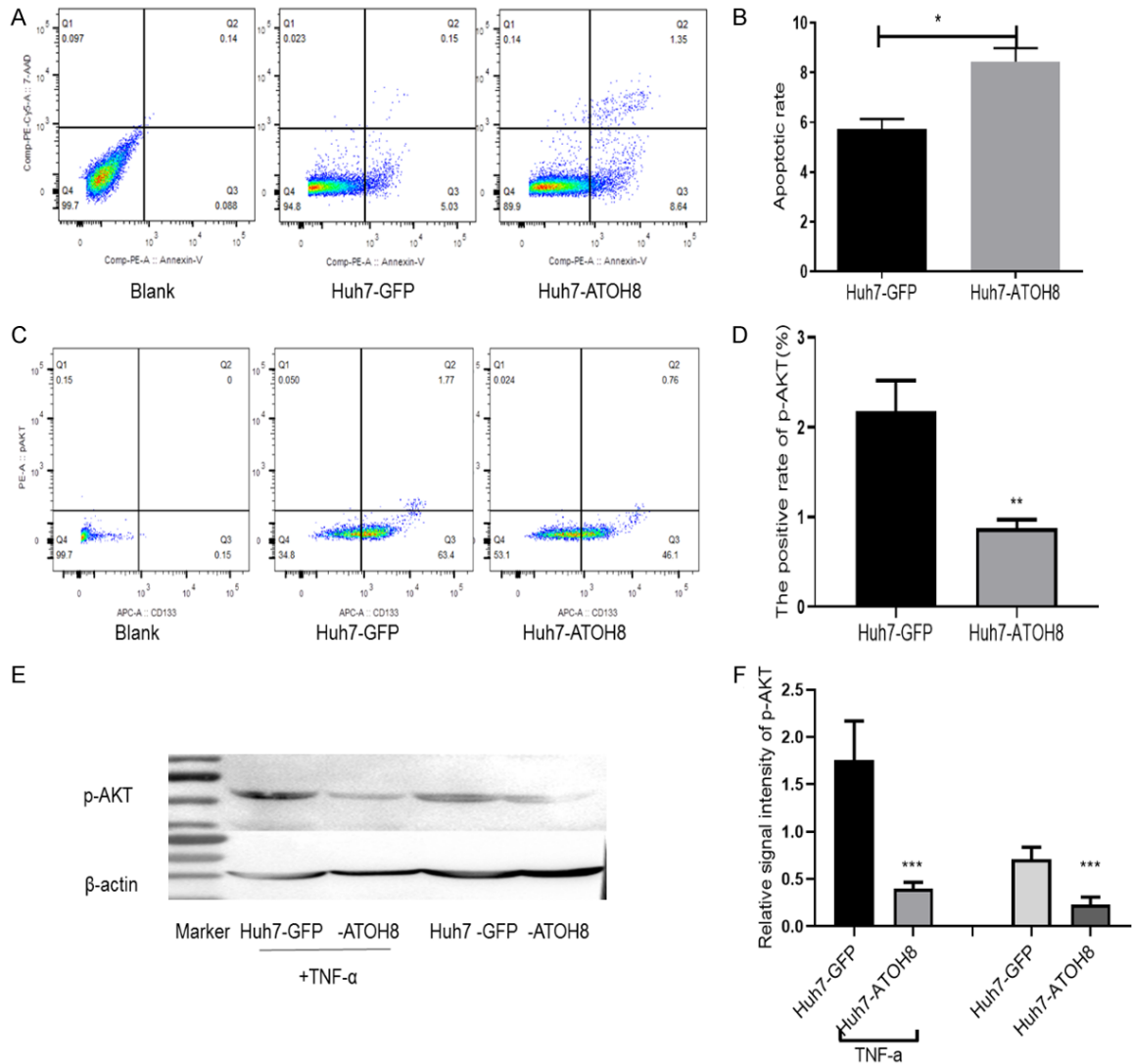

Figure 3. Overexpression of ATOH8 suppresses the phosphorylation of AKT (Ser473). A. Cell apoptosis profiles were measured by flow cytometry following treatment with or without 5-FU for 24 h. Cells in the lower right quadrant (Annexin V+/PI-) represent early apoptotic cells. B. The apoptotic rates were compared between the two groups. C. Representative flow cytometric images of intracellular staining of p-AKT (Ser473). D. The comparison of the positive rate of p-AKT. E. p-AKT (Ser473) was detected by western blot (The original lot images were shown in supplementary files). F. The relative signal intensities of p-AKT (Ser473) were analyzed by ImageJ Launcher.

short interfering RNA (siRNA). The OD450 value increased after transfection of siRNA-hATOH8 compared to the negative control group (**Figure 2E**). EMT is a major cause of tumor metastasis, so EMT-related cadherin was detected. E-cadherin decreased significantly with ATOH8 overexpression, while N-cadherin increased and the opposite effects were observed with ATOH8 silencing (**Figure 2F-H**).

Overexpression of ATOH8 increases the chemosensitivity partly by inhibiting phosphorylation of AKT (Ser473)

We explored whether ATOH8 could increase the chemosensitivity of HCC cells to 5-FU by cultivating Huh7-GFP and Huh7-ATOH8 cells with culture medium contained 5-FU (100 nM). To confirm the occurrence of apoptosis under
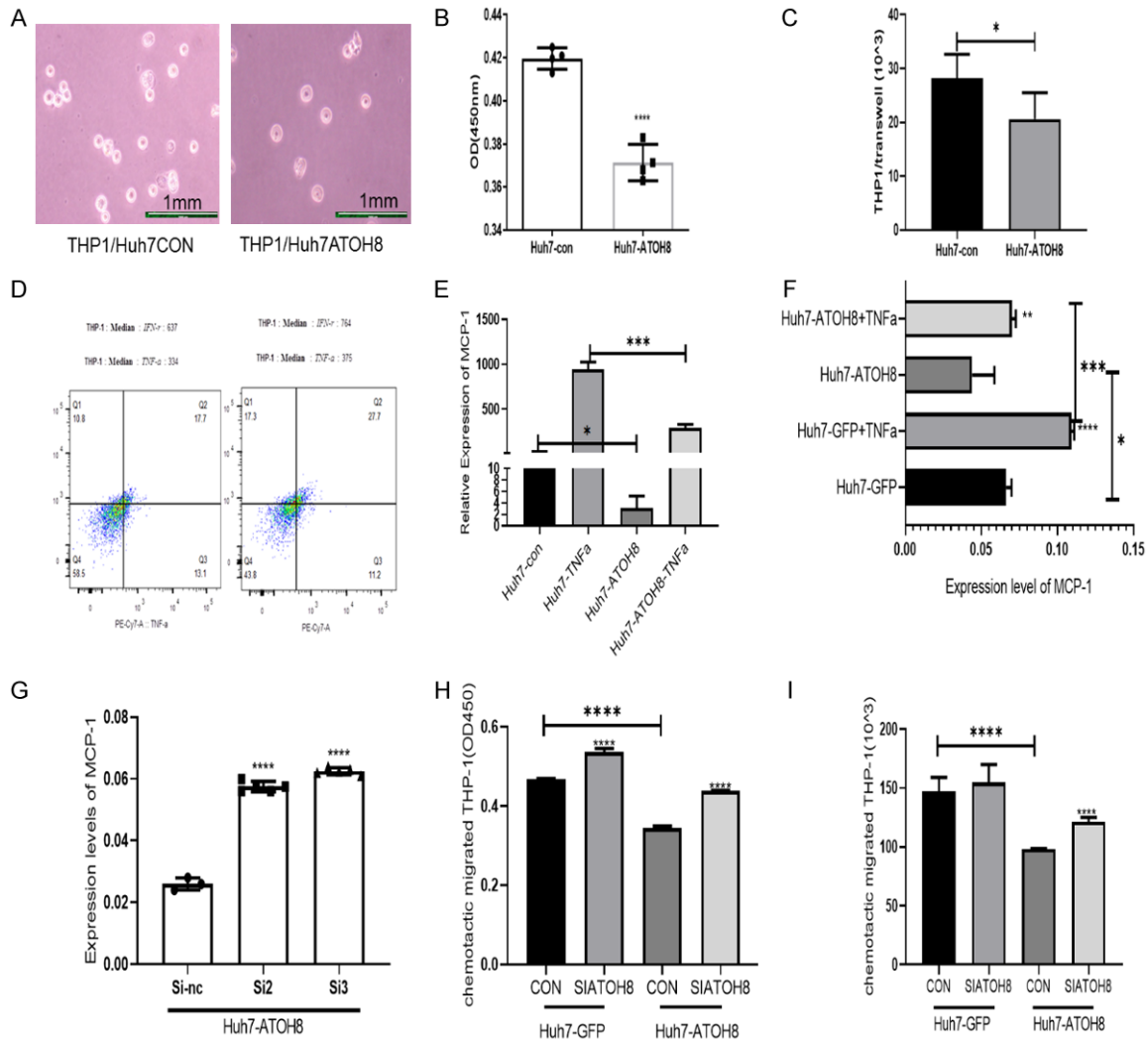
Figure 4. Upregulation of ATOH8 in Huh7 cells suppresses MCP-1 expression and chemotaxis of monocytes. A. Representative images of migrated THP-1 cells under a light microscope (The scale bar was shown in the images under a quadruple objective). B. The viability of migrated THP-1 cells was determined by using a CCK8 assay. C. The statistic analysis of the numbers of migrated THP-1 cells. D. Representative flow cytometric images of intracellular staining of TNF-α and IFN-γ. E. Quantitative RT-PCR analysis of the relative expression levels of MCP-1 in Huh7-GFP and Huh7-ATOH8 cells treated with/without TNF-a (50 ng/ml). F. The expression levels of MCP-1 in the supernatant of Huh7-GFP and Huh7-ATOH8 cells treated with/without TNF-a (50 ng/ml) were detected by ELISA. G. The expression levels of MCP-1 in the supernatant of Huh7-ATOH8 cells transfected with si-ATOH8 were detected by ELISA. H. The viability of migrated THP-1 cells was determined by using a CCK8 assay. I. The statistical analysis of the numbers of migrated THP-1 cells.

5-FU stimulation, we performed an Annexin V-PE/7-ADD double-staining assay. Apoptosis was 5.8% in the control cells and increased to 8.3% in Huh7-ATOH8 cells after 24 h of culture in media containing 100 μm 5-FU (**Figure 3A**, **3B**, P=0.03). To investigate the molecular mechanism by which ATOH8 increases the chemosensitivity, we analyzed the expression of p-AKT (Ser473) by flow cytometry (**Figure 3C**), and western blot (**Figure 3E**). As shown in **Figure 3D**, **3F**, phosphorylation of AKT (Ser473)

was inhibited in Huh7 cells overexpressing ATOH8.

Overexpression of ATOH8 modulates the microenvironment by regulating monocyte chemotaxis

To examine the chemotactic response of monocytes to Huh7 cells with/without ATOH8 overexpression, a chemotactic migration assay of monocytes was carried out in a Transwell mi-

gration chamber. The results showed that the number of chemotactic migrated monocytes was decreased when ATOH8 was overexpressed in Huh7 cells (**Figure 4A-C**), indicating that ATOH8 inhibited the chemotaxis of monocytes. Moreover, the expression of TNF-α and IFN-γ in THP-1 cells migrated in response to Huh7-ATOH8 media was higher than that in the control (**Figure 4D**). To detect the monocyte chemoattractant protein, ELISA and q-PCR were performed. ATOH8 overexpression decreased MCP-1 expression (**Figure 4E**, **4F**). To further verify chemotaxis, we also performed a chemotaxis assay in cells transfected with si-ATOH8. Increased MCP-1 levels were released by Huh7 cells after silencing ATOH8 to promote the migration of THP-1 cells (**Figure 4G-I**).

Discussion

ATOH8, belonging to group A of bHLH transcription factors, plays a crucial regulatory role in the development of many systems [12]. Many studies have reported the key role of ATOH8 in cancers [8, 9, 13]. However, its role in cancer is still controversial. For instance, it is oncogenic in colon cancer carcinogenesis but plays tumor suppressive role in nasopharyngeal cancer [8, 9]. In our study, expression and bioinformatic analyses suggest that ATOH8 is downregulated in HCC compared to adjacent tissue and that high expression of ATOH8 indicates good overall survival. This is consistent with Song's research [13]. To further investigate the function of ATOH8 in HCC, we generated ATOH8-overexpressing cells infected with lentiviruses and suppressed ATOH8 expression through transfection with ATOH8-siRNA. Overexpression of ATOH8 inhibited cell proliferation, migration, and invasion in vitro and tumorigenesis in vivo. Knocking down ATOH8 promoted cell vitality. Moreover, marker proteins associated with EMT also changed, such as N-cadherin downregulation and E-cadherin upregulation in Huh7-ATOH8 cells compared to the control. The AKT signal transduction pathway is associated with the tumor metastasis and chemoresistance [14]. Our results indicated ATOH8 inhibition of liver cancer cells can be attributed to its inhibition of the phosphorylation of the AKT signaling pathway. However, another study [15] indicated that the VEGFR2/AKT signaling pathway could upregulate ATOH8 expression in circulating colorectal cancer cells to promote colorectal can-

cer. The reason for the different roles of ATOH8 in different tumors is probably due to the fact that the bHLH domain of vertebrate ATOH8 is highly diversified. The diversification of ATOH8 may contribute delicately to the different functions in different organs [16].

Previous investigations have revealed that tumor-infiltrating monocyte/macrophage lineage cells consist of macrophages, and monocytic myeloid-derived suppressor cells exert the immunosuppressive and cancer-promoting role [17-19]. In view of the critical role of monocytes in HCC, this study further examined the effect of ATOH8 in Huh7 on THP-1 chemotaxis. Our findings demonstrated that overexpression of ATOH8 reduces monocyte chemotaxis and promotes TNF-α and IFN-γ secretion. According to the recent research monocytes cannot develop into macrophages in the liver by the Ms4a3-Cre-RosaTdT model of tracing blood monocytes [20]. We only focused on the predominantly suppressive role of monocytes in HCC, not macrophages. What's more, researches have shown that more TNF-a results in an effective killing effect on HCC cells [21] and IFN-γ could reduce the resistance to chemotherapy [22] and help to improve the patients' survival [23].

In the tumor microenvironment, chemokines can be expressed by tumor cells and other cells. Chemokines can directly target immune cells and be recruited into the tumor microenvironment [24, 25], so as to play an important role in tumor progression. MCP-1 is broadly expressed in a variety of tissue types and acts as a potent chemoattractant to recruit monocytes and macrophages to sites of inflammation [26]. The expression of MCP-1 was downregulated upon ATOH8-overexpression. Similarly, it has been shown that fast suppression of systemic MCP-1 production can reduce monocyte mobilization from the bone marrow to improve chronic inflammatory disease [27]. Our study showed that ATOH8 inhibits the chemotaxis of monocytes to manipulate the tumor immune microenvironment and plays an anti-oncogenic role in HCC by modifying MCP-1. However, we still don't know how the expression of ATOH8 in Huh7 affect the inflammatory factors secretion ability of THP-1. The expression change of MCP-1 can't explain this phenomenon. Whether there are other signal mechanisms worth further exploration.

There are also several limitations of our study. First, the molecular mechanism of ATOH8 in regulation of other chemokines in HCC remains unclear. Additionally, how ATOH8 affects MCP-1 and monocyte recruitment in vivo need further confirmation. Although our study did not involve HBV infection, the results show that ATOH8 is involved in the immunoregulation. Therefore, ATOH8 may play a vital role in antiviral immunity. All of the above unresolved issues need further research.

In conclusion, our findings show that ATOH8 expression is downregulated in HCC and leads to poor prognosis. ATOH8 can suppress Huh7 cell proliferation, migration, invasion and chemoresistance, and tumorigenic ability in nude mice, probably via inhibition of p-AKT. In addition, ATOH8 may affect the tumor microenvironment partly by regulating the chemotaxis of monocytes and the release of inflammatory cytokines in monocytes, which may provide a new perspective for the understanding of the role of ATOH8 in other diseases.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Yangqing Huang, Department of Liver Surgery, Shanghai Public Health Clinical Center, Fudan University, Shanghai 201508, China. E-mail: 17111300003@fudan.edu.cn; Dr. Jilin Cheng, Department of Gastroenterology and Hepatology, Shanghai Public Health Clinical Center, Fudan University, Shanghai 201508, China. Tel: +86-021-37990333; E-mail: fdchengjilin@qq.com

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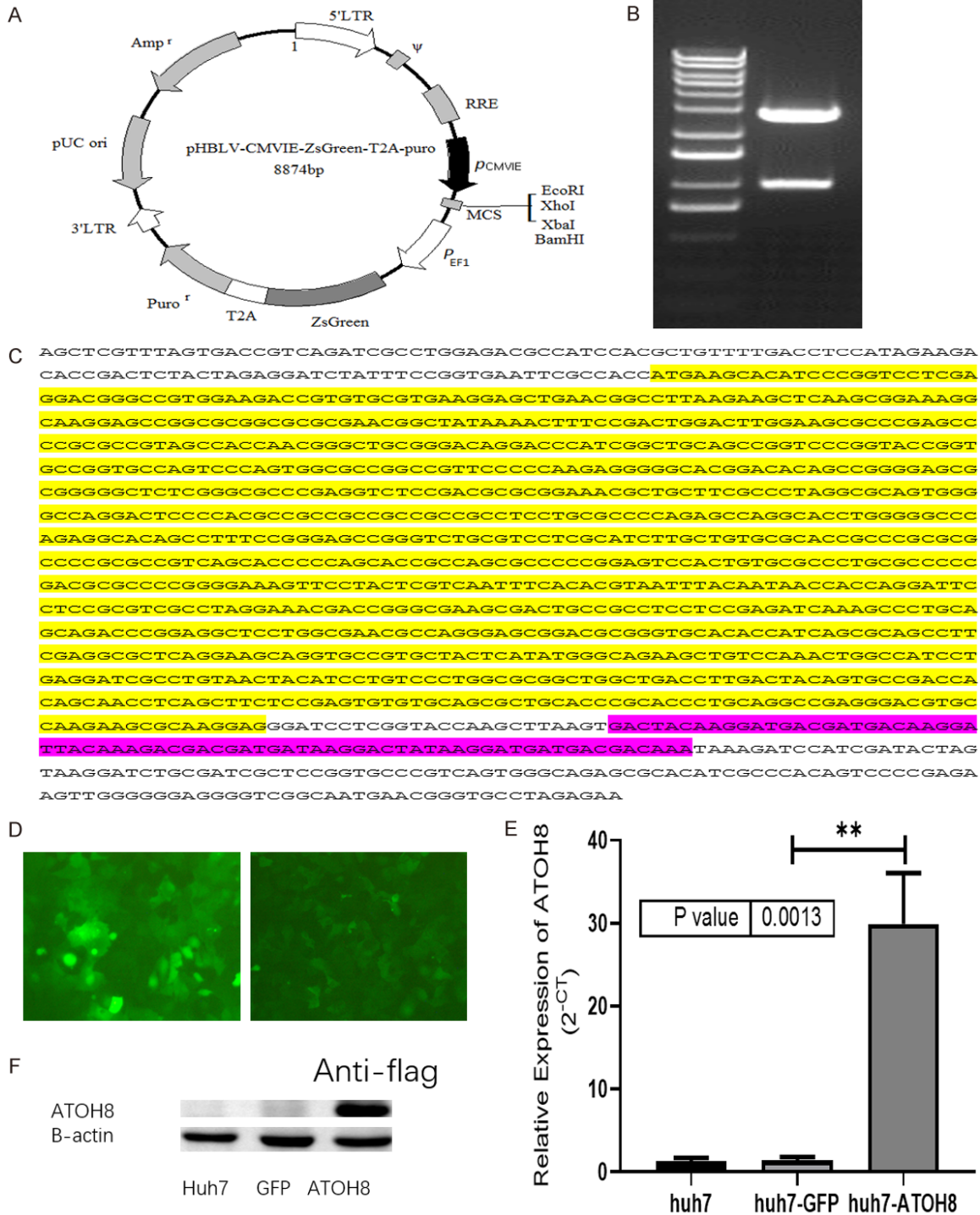
Figure S1. A. The plasmid profile of pHBLV-CMVIE. B. The electrophoretic image after enzyme digestion. C. The sequencing analysis of h-ATOH8-overexpression vector. D. The fluorescence microscope photograph of overexpressed cell lines. E. Quantitative RT-PCR analysis of the relative expression levels of ATOH8 in Huh7, Huh7-GFP and Huh7-ATOH8 cells. F. ATOH8 was detected by western blot.