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ORIGINAL ARTICLE

Xiaoaiping injection affects the invasion and metastasis of hepatocellular carcinoma by controlling AFP expression

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Abstract

Objective Xiaoaiping (XAP) is a traditional Chinese medicine that is a commonly used as an anticancer drug in clinical practice owing to its high efficiency and low toxicity. Specifically, XAP can effectively inhibit the growth of hepatocellular carcinoma (HCC). Alpha-fetoprotein (AFP) is a key HCC diagnostic marker and is closely related to certain malignant cytological behaviors of HCC. However, whether AFP expression and XAP treatment are related to the invasion and metastasis of HCC remains unclear. In the present study, we aimed to evaluate the effects and underlying mechanism of XAP on the invasion and metastasis of HCC..

Methods Using a cell scratch assay, Transwell technology, and western blotting we detected the different invasion and metastatic abilities of Hep3B cells in XAP treatment and blank control groups. This allowed comparison of the invasion and metastatic abilities of Hep3B cells with differing levels of AFP expression. AFP mRNA sequencing technology was used to analyze the mechanism of tumor invasion and metastasis associated with AFP and XAP treatment.

Results Cell invasion and metastasis abilities in the XAP group were significantly lower than those in the control group ($P < 0.05$). Additionally, compared to the control group, the expression of AFP significantly decreased after XAP treatment ($P < 0.05$). The ability of Hep3B cells to invade and metastasize was promoted when AFP expression was up-regulated, whereas it was inhibited when AFP was silenced. XAP injection and AFP regulate the invasion and metastatic ability of HCC by affecting matrix metalloproteinases (MMPs).

Conclusion XAP injection inhibits the invasion and metastatic ability of HCC by influencing the expression of AFP; additionally, this inhibition of AFP is achieved by affecting MMPs.

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Cancer is a globally prevalent malignant disease that threatens quality of life, with a corresponding increase in cancer-related incidence and mortality every year [1]. Primary hepatocellular carcinoma (HCC), which is the second most common cancer worldwide in terms of tumor mortality, is one of the ten most malignant tumors in the world. There are more than 500,000 new HCC-related cases and deaths worldwide every year, of which approximately 51% occur in China [2, 3]. At present, HCC treatments have been developed from surgical and radiotherapy techniques, interventional chemoembolization, and novel molecular targeted drugs [4]. However, due to the complexity of the occurrence and development of HCC, singular or traditional treatment methods cannot satisfy clinical needs. Surgical treatment and radiotherapy aim to avoid the complications

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associated with chemotherapy, such as physical trauma and adverse side effects [5, 6]. In fact, the primary reason why HCC has become a long-term problem in humans is that it is a vascular-rich tumor with high vascular invasiveness with tendency for distant metastasis [7]. AFP is a specific diagnostic marker of HCC. It has been widely established in clinical data that patients with high levels of AFP protein and/or gene have significantly higher postoperative tumor metastasis and recurrence rates than those with lower AFP expression [8]. Moreover, AFP has the ability to promote HCC cell invasion *in vitro* by activating the PI3K/Akt/mTOR signaling pathway [9].

At present, traditional Chinese medicine (TCM) are widely researched in the study of anti-tumor therapeutics because of their unique advantages, such as no obvious side effects, high efficiency, and low toxicity [10]. Therefore, an increasing number of people have turned their attention to TCM, which can regulate the immune function of the body while demonstrating anti-tumor characteristics. Moreover, TCM does not simply kill tumor cells, but can strongly influence their metastatic and invasion abilities [11, 12]. Xiaoaiping (XAP) injection, a TCM preparation extracted from *Marsdenia tenacissima* (MT), can improve quality of life, strengthen immune function, and effectively prolong the survival of tumor patients [13]. Previous studies have indicated that XAP has remarkable curative effects on gastric cancer, lung cancer, esophageal cancer, osteosarcoma, and many types of malignant tumors, including HCC; in particular, XAP inhibits cellular proliferation and induces cell apoptosis in these tumors [14, 15]. Dai et al. showed that after treatment of A20 mouse lymphoma with XAP, the production of angiogenesis-related molecules, such as vascular endothelial growth factor and matrix metalloproteinases (MMPs), was significantly reduced, and angiogenesis in tumor tissues consequently decreased [16]. It has been confirmed by *in vitro* cell experiments that XAP can inhibit growth and promote apoptosis of HepG2 and Bel7402 cells in human HCC, thus achieving potential efficacy in clinical treatment of HCC [17]. However, its corresponding effectiveness and mechanism of action in HCC metastasis remains unclear.

The present study was designed to investigate the effects of XAP on the migration of human HCC Hep3B cells and to explore the role of AFP in the anti-metastatic effect of XAP.

Materials and methods

Chemicals and regents

Hep3B Cell lines were purchased from China typical species Conservation Center (Wuhan, China); the cells were cultured in medium consisting of Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin in an incubator at 37 °C with 95% air, and 5% CO_2. XAP injection was purchased from Nanjing Shenghe Pharmaceutical Co., Ltd. (No. Z20025868). DMEM medium and FBS were purchased from Hyclone Corporation, USA, Trizol was purchased from Invitrogen, and a reverse transcription kit was purchased from Fermentas.

RNA interference

Hep3B cells in the logarithmic growth phase were cultured in DMEM containing 5% FBS; these cells were seeded in a 6-well plate. We selected anti-AFP-specific siRNA-expressing vectors (AFP-siRNA) directed at the 923–944 region of the AFP gene [18]. Transfection of AFP-siRNA vectors and the control virus vector into Hep3B cells was induced using Lipofectamine 2000 (Invitrogen, USA) once the confluency of the cells reached approximately 65%. The cells were cultured for five days and AFP expression was detected using western blotting.

Generation of an AFP-expressing construct

AFP-expressing cells (pcDNA3.1-AFP) [18] were constructed by lentiviral infection as previously described. Hep3B cells were transfected with the control virus vector and packaging plasmids (pcDNA3.1-AFP) using Lipofectamine 2000 (Invitrogen, USA). The cells were incubated with the plasmid-lipid complex for 24 h and western blot analysis was performed to detect AFP levels.

Western blotting

Western blotting was performed as previously described. Briefly, A549 cells were washed twice with phosphate-buffered saline and lysed in radioimmunoprecipitation assay (RIPA) buffer. Cell lysates were incubated at 4 °C for 30 min, and cellular debris were pelleted by centrifugation at 15000 × g for 15 min at 4°C. Total protein was quantified using the BCA Protein Assay Reagent Kit with bovine serum albumin as a standard. Equal amounts (50 μg) of protein were loaded into each lane of a 12% SDS-PAGE gel, followed by transfer to a polyvinylidene fluoride membrane (Bio-Rad, USA) on a semi-dry transfer apparatus (Bio-Rad). After blocking with 5% non-fat milk for 1 h at room temperature, the membrane was incubated with specific antibodies overnight at 4°C. After washing, horseradish peroxidase–linked anti-mouse IgG was used as the secondary antibody and incubated with the membrane for a further 1 h at room temperature. Protein bands were detected using ECL Western blotting detection reagents (Amersham Biosciences, USA). All antibodies used in this experiment were purchased from Santa Cruz Biotech.

Scratch test

Cell migration was evaluated using a scratch test. The cells were grown to 80% confluency in 12-well microplates before scratching. Cell images were captured using a light microscope at 0, 24, and 48 h after treatment. The scratch area was calculated using ImageJ software.

Transwell assay

The migration assay for Hep3B cells was performed using Transwell membranes (Corning, USA) coated with Matrigel (BD Biosciences, USA). DMEM containing 10% FBS was placed in a 24-well chamber. The invading cells were removed from the top well using a cotton swab. After washing three times with PBS, the cells were fixed for 30 min and stained with 0.1% crystal violet for 20 min. The cells were incubated at 37 °C for 24 h. Migrating cells attached to the subventricular surface were observed under a microscope (×200 magnification). The number of cells entering the chamber were counted in five random fields to evaluate cell invasion and migration. The experiment was repeated thrice. Invasion rate was determined as follows: Invasion rate (%) = number of invasive cells in the experimental group/number of invasive cells in the control group × 100%.

Statistical analysis

Statistical analysis was performed using SPSS software (version 22.0; IBM Corp., Armonk, NY, USA), and the results were expressed as mean ± standard deviation ($\bar{x}$ ± s); P<0.05 was considered statistically significant. Image-pro plus 6.0 (Media Cybernetics, Inc., USA) was used to count the number of cells and calculate the scratch areas.

Results

XAP can inhibit the invasion and metastasis of HCC

The migration abilities of Hep3B cells injected with XAP (80 μL/mL) and the control group were detected by the cell scratch test and Transwell metastasis assay. The corresponding results showed that the cell migration and invasion abilities of the XAP and control groups were significantly different (P< 0.05). Compared to that in the control group, the migration distance length and the number of invading cells in the XAP-treated group were significantly lower (both P<0.05). Therefore, these results revealed that XAP injection impaired HCC cell migration and invasion abilities (Fig. 1).

XAP can inhibit the expression of AFP

Next, we detected the influence of XAP injection on the expression of AFP protein using western blotting. The XAP-treated Hep3B/HepG2 cells exhibited a downregulated expression of AFP protein (Fig. 2).

AFP overexpression promotes invasion and metastatic ability of Hep3B cells

The migration distances of the two groups of cells were observed under a microscope at 0, 24, and 48 h after transfection of Hep3B cells with the AFP-expressing lentivirus vector (pcDNA3.1-AFP). The migration distance in the AFP overexpression group was significantly smaller than that in the control group (P < 0.05). The corresponding Transwell assay results were consistent with those of the scratch test in that the number of cells penetrating the membrane in the AFP overexpression group was significantly higher than that in the control group. Overall, these results indicated that cell invasion and metastasis were enhanced when AFP expression was upregulated (Fig. 3).

Silencing AFP expression reduces the invasion and metastasis ability of Hep3B cells

Compared with that in the control group, the migration distance of cells in the silencing shRNA-AFP group was significantly higher (P < 0.05) and the number of cells in shRNA-AFP group was significantly decreased (P < 0.05). Therefore, these results demonstrated that the metastatic ability of Hep3B cells decreased with silencing of AFP expression (Fig. 4).

XAP and AFP control HCC invasion and metastasis by affecting the expression of MMPs

Using Western Blotting, we determined that the expression levels of MMP-2 and MMP-7 in the two cell lines treated with XAP were significantly decreased compared to those in the controls (P<0. 05) (Fig. 5).

Discussion

HCC accounts for approximately 90% of primary liver cancers and is prone to intrahepatic and extrahepatic metastasis. Radical resection of HCC is currently the most effective treatment; however, several studies have determined that even with radical surgery, 50% of patients still develop metastases within 5 years, which has been attributed to the vascular-enrichment characteristics of HCC [7, 19]. Therefore, exploring the mechanisms of HCC invasion and metastasis has important clinical significance in the prevention and treatment of this cancer. XAP, one of the most popular TCM drugs for HCC treatment, has been shown to inhibit the growth and proliferation of many types of tumor cells, including HCC, and possesses unique clinical anti-tumor effects [14, 15]. Although XAP injection has shown promising cytotoxicity against primary tumors, its effect on cancer metastasis remains unknown. The results from the current study demonstrated that the invasion and metastasis ability of Hep3B cells treated

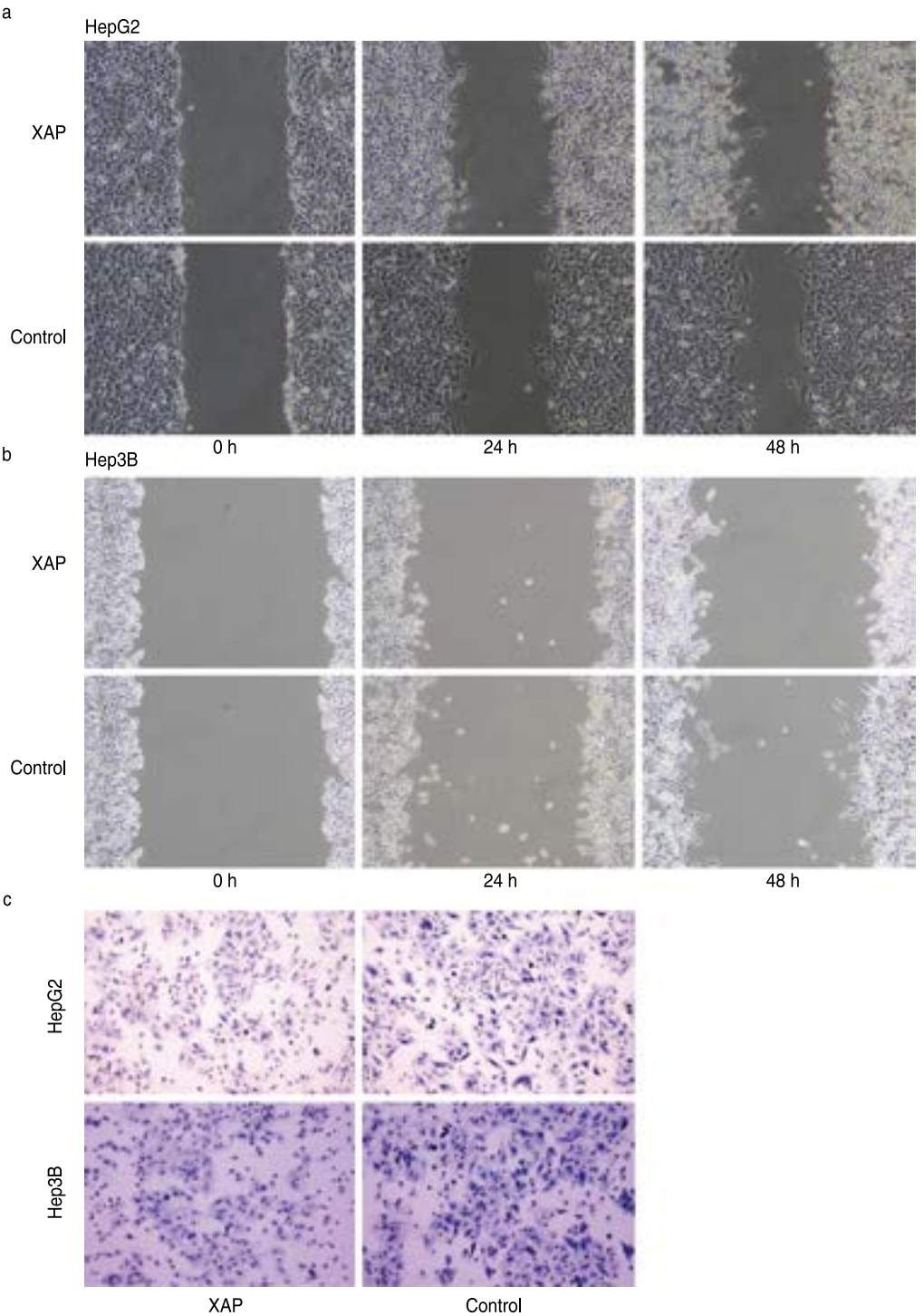

Fig. 1 Xiaoaiping (XAP) can inhibit the invasion and metastasis of hepatocellular carcinoma (HCC). (a, b) The migration distance of the cell scratch between XAP group and the control group are measured at 0 h, 24 h, and 48 h; (c) Comparison of metastatic ability between XAP and control group cells in Hep3B and HepG2 using a Transwell metastasis assay. The corresponding results indicate that the number of cells in the Hep3B/XAP and HepG2/XAP groups were significantly lower than in the corresponding controls ($P < 0.05$)

with XAP injection was significantly lower than that of the control group, suggesting that XAP may exert its clinical anti-tumor effects by influencing the distant metastasis of HCC; these findings, therefore, address our previous questions. These findings are consistent with a previously reported conclusion that XAP has an inhibitory effect on the invasion and metastasis of A549 cells through regulation of the CCR5–CCL5 axis, Rho C,

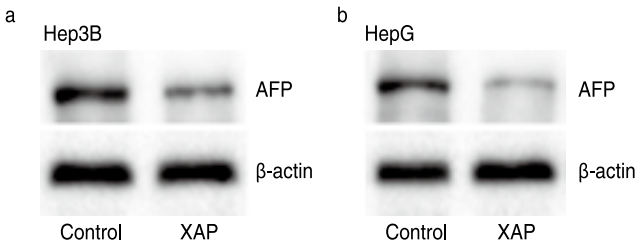

Fig. 2 Xiaoaiping (XAP) inhibits the protein expression of alpha-fetoprotein (AFP). The expression of AFP in the XAP group was significantly lower than that in control group

with AFP expression vectors, when compared with control cells transfected with an empty vector ($P < 0.05$), indicating that AFP can promote HCC cell invasion *in vitro* [17]. Additionally, Zhu has reported that AFP induces the expression of CXCR4 by activating the AKT/mTOR signaling pathway, thus enhancing the ability of liver cancer invasion and metastasis [20]. These findings are consistent with our experimental results, which demonstrate that the expression level of AFP influences the invasion and metastasis of Hep3B cells. Further, we confirmed that overexpression of AFP promoted invasion

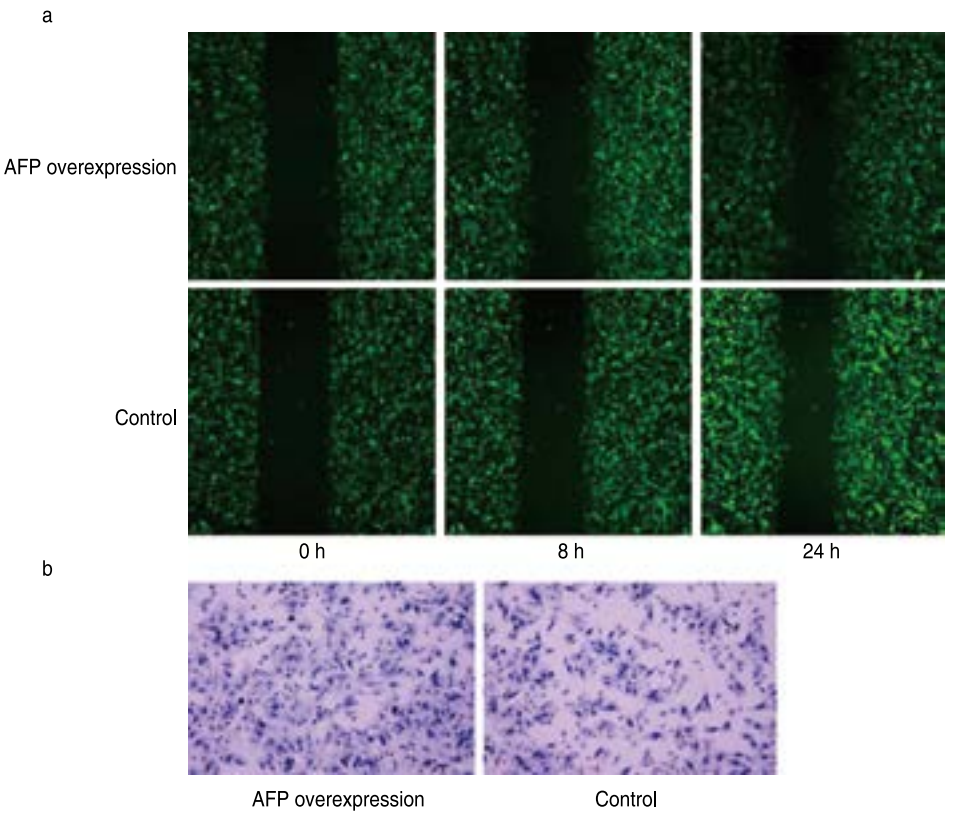

Fig. 3 Effect of alpha-fetoprotein (AFP) overexpression on invasion and metastasis of hepatocellular carcinoma. (a) Overexpression of AFP enhances the ability of cell invasion and metastasis of Hep3B, illustrated by a cell scratch test. The migration distance in the AFP overexpression group is significantly shorter than that in the control group ($P < 0.05$); (b) Migration of Hep3B cells after enhancing AFP expression using a Transwell chamber assay. When AFP is up-regulated, the number of cells significantly increases ($P < 0.05$)

and phosphorylated FAK [21].

To date, prior studies have shown that AFP plays a critical role in the effects of anti-tumor drugs in HCC cells [22]; further, AFP expression has been positively correlated with HCC invasion and metastasis [23, 24]. Previous studies have shown that the serum AFP level is closely related to the invasion and metastasis of HCC. Specifically, in our previous study, we confirmed that when the serum AFP level reached above 400 μg/L, HCC patients possessed a higher risk of tumor invasion and postoperative metastasis [8]. According to Wang, the migratory capacity of HCC cells can be significantly enhanced following transfection and metastasis of Hep3B cells, whereas the migration ability was inhibited by AFP silencing using a Transwell assay and scratch test.

The results from the present study explore the mechanisms of XAP treatment and suggest that XAP inhibits the expression of AFP protein and can weaken the invasion and metastasis of HCC cells. Since MMPs are the only known matrix degradation enzymes of interstitial collagen that can degrade all components of the extracellular matrix (ECM) released from polysaccharides, we examined the influence of XAP on

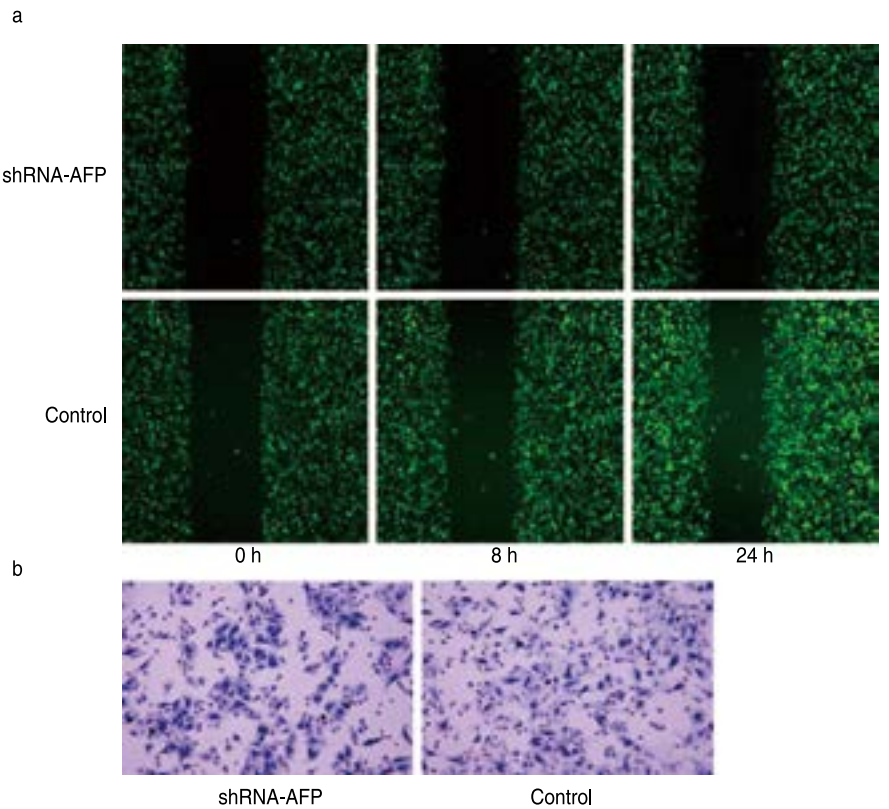

Fig. 4 The results showed that the metastatic ability of Hep3B cells was reduced after AFP expression was silenced. (a) The cell scratch test showed that alpha-fetoprotein (AFP) depletion resulted in reduced cell migration and invasion; (b) The cell migration in Hep3B cells after silencing AFP by using the transwell chamber experiment among the two groups

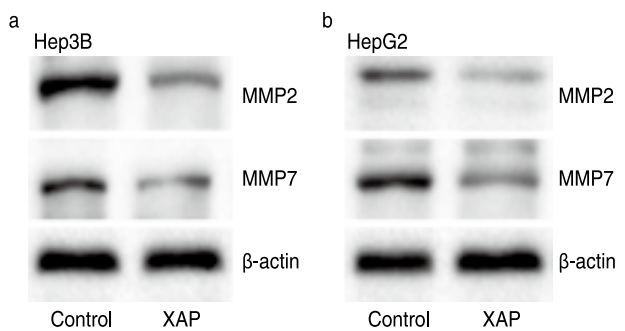

Fig. 5 Expression of MMP-2 and MMP-7 was significantly reduced in two cell lines treated with XAP. (a) Compared with the control group, XAP could down-regulate the expression of MMP-2 and MMP-7 expressions in Hep3B cells; (b) Compared with the control group, XAP could down-regulate the expression of MMP-2 and MMP-7 expressions in HepG2 cells

the expression of certain members of the MMP family by using western blotting. Consequently, it was observed that the expression of MMP-2 and MMP-7 proteins was downregulated in XAP-treated cells. It is well established that MMPs can participate in the regulation of the tumor microenvironment, predominantly through both proteolytic and non-proteolytic processes; consequently, these enzymes play an important role in ECM circulation, cell growth, inflammation, angiogenesis, and tumor cell migration [25]. Since the discovery of the first MMP in 1964, MMP-1, the number of MMP family members has increased to 26, with high homology among their corresponding genes [26]. Blood analysis of patients with liver disease and liver cancer demonstrated that the MMP-2 expression was significantly higher than normal in these groups [27]. In addition, upregulation of MMP-2 expression and activity has been shown to play a key role in a variety of human cancers with metastatic ability [28]. MMP-2 is the main enzyme in the MMP family; specifically, MMP-2 degrades type IV collagen and participates in the degradation of the ECM and basement membrane, thereby playing an important role in tumor invasion and metastasis [29, 30]. MMP-7 is a secreted protein involved in the destruction of the ECM in many cancers and can promote the metastasis of liver cancer [31, 32]. Lynch reported that abnormal expression of MMP-7 may initiate of cellular processes and promotion of cellular migration by converting the cell adhesion protein E-cadherin into a soluble form in tumor cells; this can lead to the freeing of cancer cells from large tumor tissues, improving cell migration [33]. This understanding highlights the understanding that the expression levels of MMP-2 and MMP-7 are important indices for evaluating tumor

invasion and metastasis. Therefore, this is a reasonable explanation for the reduced expression levels of MMP-2 and MMP-7 observed after XAP treatment in our study, which were accompanied by weakened invasion and metastatic ability of liver cancer cells.

Conclusion

XAP injection inhibits the invasion and metastatic ability of HCC by influencing the expression of AFP; further, the corresponding inhibition of AFP is achieved by XAP-mediated downregulation of MMP-2 and MMP-7 expression.

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Conflicts of interest
The authors declare that they have no conflicts of interest.

Author contributions
Not applicable.

Data availability statement
All data generated or analyzed during this study are included in this published article (and its supplementary information files).

Ethical approval
Not applicable.

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