Original Paper

The Effects of *Angelica Sinensis* Polysaccharide on Tumor Growth and Iron Metabolism by Regulating Hepcidin in Tumor-Bearing Mice

Feng Ren^{a,b} Jian Li^{c,d} Yanglin Wang^a Yongxia Wang^a Sijia Feng^e
Zhiqing Yuan^a Xinlai Qian^a

^aDepartment of Pathology, Xinxiang Medical University, Xinxiang, ^bHenan Collaborative Innovation Center of Molecular Diagnosis and Laboratory Medicine, Xinxiang Medical University, Xinxiang, ^cDepartment of Forensic Medicine, Xinxiang Medical University, Xinxiang, ^dDepartment of Pathology, School of Basic Medical Sciences, Southern Medical University, Guangzhou, ^eDepartment of pathology, the Third Affiliated Hospital of Xinxiang Medical University, Xinxiang, China

Key Words

Cancer • Iron load • *Angelica sinensis* polysaccharide • Hepcidin

Abstract

Background/Aims: Iron plays a fundamental role in cell biology and its concentration must be precisely regulated. It is well documented that excess iron burden contributes to the occurrence and progression of cancer. Hepcidin secreted by liver plays an essential role in orchestrating iron metabolism. In the present study, we aimed to investigate the ability of *angelica sinensis* polysaccharide (ASP) to decrease iron burden in tumor-bearing mice and the mechanism of ASP regulation hepcidin expression. **Methods:** Western blot, RT-PCR, immunohistochemistry (IHC), and enzyme-linked immunosorbent assay (ELISA) were used to detect the regulation of hepcidin and related cytokines by ASP. The role of ASP in tumor proliferation was investigated using in vivo assays. Iron depositions and iron concentrations in organs were determined by hematoxylin-eosin (H&E) staining and atomic absorption spectrophotometer. **Results:** We found that ASP could inhibit tumor growth in mice xenografted with 4T1 and H22 cancer cells. In vivo experiments also showed that ASP could potentially regulate hepcidin expression in liver and serum and decrease iron burden in liver, spleen and grafted tumors in mouse model. Treatment with ASP in hepatic cell lines reproduced comparable results in decreasing hepcidin as in mouse liver. Furthermore, we found that ASP markedly suppressed the expression of interleukin-6 (IL-6), JAK2, p-STAT3, and p-SMAD1/5/8 in liver, suggesting that JAK/STAT and BMP-SMAD pathways were involved in the regulation of hepcidin expression by ASP. We also

Feng Ren and Jian Li contributed equally to this work.

Xinlai Qian
and Feng Ren

Department of Pathology
Xinxiang Medical University, Xinxiang 453003 (China)
Tel. +86-373-3029956 E-Mail 18937332097rf@sina.com / renfeng@xxmu.edu.cn

found down-regulation of iron-related cytokines in ASP treated mice. **Conclusion:** The present study provides new evidence that ASP decreases hepcidin expression, which can reduce iron burden and inhibit tumor proliferation. These findings might aid ASP developed as a potential candidate for cancer treatment in patients with iron overload.

© 2018 The Author(s)
Published by S. Karger AG, Basel

Introduction

Iron is involved in a variety of crucial biological processes including energy generation, oxygen transport, DNA synthesis, and enzymatic processes, and acts as an essential element for the majority of the organisms [1]. Iron overload is a severe condition, and increasing evidence indicated that iron overload may contribute to tumor initiation, behavior and recurrence [2-5]. Hepcidin, a peptide hormone is secreted mainly by the liver and function as a key regulator of iron homeostasis [6]. Previous studies have revealed that increased serum hepcidin was related to multiple cancers, such as breast cancer, myeloma, renal cell carcinoma and prostate cancer [7-10]. Hepcidin is homeostatically regulated by iron and erythropoietic activity as well as inflammatory cytokines [9, 11]. Hepcidin inhibits iron absorption from the duodenum and iron egress from macrophages and hepatocytes through binding and inducing degradation of iron exporter ferroportin [12, 13]. Accumulated evidences suggest that therapeutic regimen targeting the hepcidin-ferroportin axis could have important clinical implications for cancer patients with iron overload.

Angelica sinensis is broadly used as Chinese herbal medicine or food in Asian countries such as China, Japan and Korea [14]. ASP is the major bioactive component extracted from roots of *angelica*. Recently, studies have demonstrated that ASP has various biological functions, including immunomodulation, anti-tumor activity, and hematopoiesis [15, 16]. It was reported that ASP could regulate hepcidin expression and inhibit tumor growth in H22 tumor-bearing mice [17]. However, whether ASP has the ability to decrease iron burden in tumor-bearing mice and the mechanism of ASP regulation hepcidin expression are still unknown. In this study, we confirmed that ASP could potentially regulate hepcidin expression and inhibited tumor growth in mice xenografted with 4T1 and H22 cancer cells. Furthermore, we firstly investigated the role of ASP on decrease iron burden in liver, spleen and grafted tumors in mice and explored the potential mechanism of ASP regulating hepcidin via the Janus-kinase/ signal transducer and activators of transcription (JAK/STAT) and bone morphogenetic protein-small mothers against decapentaplegic (BMP-SMAD) signaling pathways.

Materials and Methods

Cell lines and cell culture

Human normal liver cell line (L-02), hepatocellular carcinoma cell line (HepG2) and mouse breast cancer cell line (4T1) were purchased from the cell bank of Chinese Academy of Sciences. Mouse hepatocellular carcinoma cell line (H22) was obtained from China Center for Type Culture Collection. All cells were authenticated by short tandem repeat (STR) profiling before receipt and were propagated for less than 6 months after resuscitation. These cells were maintained in DMEM (Hyclone, Logan city, USA) supplemented with 10% fetal bovine serum (FBS) and incubated in a 5% CO₂ humidified atmosphere at 37°C. L-02 and HepG2 cells were treated with ASP in different time or concentration. ASP with >98% purity was purchased from Ci Yuan Biotechnology Co., Ltd. Shanxi (Xian, China).

Animals

Balb/c mice (4-5 weeks old) purchased from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). Mice didn't take any treatment as the blank control group. For *in vivo* tumor growth assay, xenograft tumors were generated by subcutaneous injection of 2×10⁶ 4T1 cells or H22 cells. Tumor-bearing mice were randomly divided into 3 groups: the control group; the CTX group and the ASP group. After 6 days of tumor inoculation, the CTX group was intraperitoneally injected with cytoxan (CTX, Jiangsu Hengrui Medicine Co., Ltd., Lianyungang, china) at 25 mg/kg once daily for 14 days and the ASP group were intraperitoneally

injected with ASP at 100 mg/kg once daily for 14 days. The control group was intraperitoneally injected with the same volume of 0.9% normal saline (NS). Dosages and time were determined according to preliminary experiments [18]. Mice body weight and tumor size were monitored every other days.

At the end of the experiment, all mice were humanely euthanized, and the blood samples were collected into Eppendorf tubes without anticoagulant from the angular vein. After 4h standing at room temperature, serum was obtained after centrifugation at 12000 rpm min⁻¹ for 10 min, and then the supernatant was collected and stored at -20°C. Liver, spleen and tumor were removed, weighed, rinsed and subjected to following analysis.

All animal experiments were approved by the Use Committee for Animal Care and performed in accordance with institutional guidelines.

Measurement of iron concentration in mice

Iron concentration in mice serum was assayed by automatic biochemistry analyzer (SIMENS Advia 2400). Iron depositions in liver, spleen, and tumor were determined by atomic absorption spectrophotometer (AA240FS series by Varian Australia Pty Ltd.).

Histological analysis and Immunohistochemical analysis

Liver, spleen, and tumor of mice in each group were harvested and paraffin embedded. 5µm-thick sections were processed with H&E staining for observation of iron depositions. For IHC, liver specimens were incubated with rabbit polyclonal hepcidin-25 antibody (Abcam, Cambridge, UK), tumor specimens were incubated with rabbit Ki67 primary antibody (BD Biosciences, San Jose, CA, USA) at 4°C overnight. Then these were incubated with HRP-conjugated secondary antibodies (Cell Signaling Technology, Beverly, USA) at room temperature for 2h. Immunohistochemically stained sections were reviewed and evaluated by two independent pathologists. All of the slides were observed under a Nikon light microscope (Nikon Corporation, Tokyo, Japan) and representative photographs were naptured.

Enzyme-linked immunosorbent assay

The concentration of Hepcidin (USCN Life Co., Houston, TX, USA), ferritin, Tf, TfR1, TfR2 and IL-6 (Elabscience Biotechnology Co., Ltd, Wuhan, china) in mice serum was determined by ELISA according to the manufacturer's instructions.

RT-PCR and Western blot assays

The assay of RT-PCR was carried out as described previously [19]. Primer sequences were as follows: 5'-CCTGACCAGTGGCTCTGTTT-3', 5'-CACATCCCACACTTTGATCG-3' for hepcidin; 5'-GTGGGGCGCCCCAGGCACCA-3', 5'-CTCCTTAATGTACGCACGATTTC-3' for GAPDH.

Protein lysates were prepared, subjected to SDS-PAGE, transferred onto PVDF membranes and blotted according to standard methods, using anti-hepcidin (Alpha Diagnostic International, San Antonio, USA), anti-p-STAT3 (Invitrogen, Carlsbad, USA), anti-IL-6, anti-p-SMAD1/5/8 (Santa Cruz Biotechnology, Santa Cruz, USA), and anti-JAK2 (Cell Signaling Technology, Beverly, USA), GAPDH and anti-β-actin rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, USA).

Statistical analysis

Data were presented as mean ± standard deviation (SD) for normal distribution. Groups were compared by one-way Analysis of variance (ANOVA) and multiple comparisons by LSD-t test by SPSS 21.0 (IBM SPSS for Windows, Version 21.0; IBM Corporation, Armonk, NY, USA). Outliers were excluded with larger or smaller than 2 SD. P < 0.05 was considered significant.

Results

ASP inhibits tumor growth in vivo

In order to investigate the effects of ASP on tumor growth in tumor-bearing mice, volume and weight of tumors were monitored every other day after 4T1 and H22 cells inoculated subcutaneously. As shown in Fig. 1A-C, the tumors in the CTX and ASP groups were smaller

in size than those in the saline treated tumor-bearing control group for both 4T1 and H22 cells xenografted mice. Furthermore, immuno- histochemistry (IHC) confirmed that tumors of the control group displayed significantly higher Ki-67 expression than those in the ASP group (Fig. 1D, E). These results indicated that ASP displayed anti-tumor effects and could inhibited tumor growth *in vivo*.

ASP regulates iron burden in multiple organs, grafted tumors, and serum in vivo

One of the consequences of iron overload is the deposition of hemosiderin in the liver, spleen, and tumor tissues. Hemosiderin is the compound formed of broken hemoglobin, ferric oxide (unused iron) and ferritin. Iron depositions in liver, spleen, and tumor tissues were determined by hematoxylin-eosin (H&E) staining. The liver and spleen sections from the saline treated tumor-bearing control group mice showed increased hemosiderin deposition compared to the tumor free blank group. However, the liver, spleen, and tumor sections derived from the ASP-treated mice showed fewer hemosiderosis than those of the control group (Fig. 2A, C). Iron concentrations in these organs were detected by atomic absorption spectrophotometer. Results showed that in 4T1 tumor-bearing mice the iron concentration of liver and spleen in control group mice increased 2.71 and 4.55-fold compared to the blank

Fig. 1. ASP inhibits tumor growth in 4T1 and H22 tumor-bearing mice. 4T1 cells and H22 cells were subcutaneously injected into the right axillary of 4-5-week old Balb/c mice. (A) The images of tumors from 4T1 and H22 tumor-bearing mice. (B) The final tumor weights of 4T1 and H22 tumor-bearing mice were measured. Data points are presented as the mean tumor weight $\pm$ SD. (C) The tumor volumes of 4T1 and H22 tumor-bearing mice were measured on the indicated days. Data points are presented as the mean tumor volume $\pm$ SD. (D) Histopathological analyses of tumor growth in mice xenografted with 4T1 cancer cells. The tumor sections were subjected to IHC staining using an antibody against Ki-67. The magnification is 400x. (E) Histopathological analyses of tumor growth in mice xenografted with H22 cancer cells. The tumor sections were subjected to IHC staining using an antibody against Ki-67. The magnification is 400x. Error bars represent mean $\pm$ SD from three independent experiments. (* P <0.05, ** P <0.01, or *** P <0.001).

Fig. 2. ASP adjusts the iron concentrations in serum and tissues of 4T1 and H22 tumor-bearing mice. Analysis of iron concentration and deposits in serum, liver, spleen, and tumor of 4T1 and H22 tumor-bearing mice following 14 days of ASP therapy. (A) Representative histological analysis of iron deposits in liver, spleen, and tumor of 4T1 tumor-bearing mice. Specimens were stained with H&E staining. Brown hemosiderin indicated the distribution of iron depositions. The magnification is 400x. (B) In 4T1 tumor-bearing mice, iron concentration in liver, spleen, and tumor was assayed by atomic absorption spectrophotometer and iron concentration in serum was assayed by automatic biochemistry analyzer. (C) Representative histological analysis of iron deposits in liver, spleen, and tumor of H22 tumor-bearing mice. The magnification is 400x. (D) In H22 tumor-bearing mice, iron concentration in liver, spleen, and tumor was assayed by atomic absorption spectrophotometer and iron concentration in serum was assayed by automatic biochemistry analyzer.

group ($P < 0.001$). After treatment with ASP, the iron concentration in liver, spleen and tumor tissues were reduced by 38.53%, 32.88% and 55.89% in comparison to the saline treated control group, respectively ($P < 0.001$) (Fig. 2B). Comparable results were observed in H22 tumor-bearing mice. Iron concentration of liver and spleen in control group increased 3.36 and 7.92-fold when compared to the blank group, respectively ($P < 0.001$). After treatment

of ASP the iron concentration in liver, spleen and tumor reduced by 36.99%, 41.71% and 35.08% compared to the control group, respectively ($P < 0.001$) (Fig. 2D). We next analyzed the influence of ASP on serum iron concentration of 4T1 and H22 cells xenografted mice. The serum samples were separated from angular vein of these mice. Then the iron concentration in serum was assayed by automatic biochemistry analyzer. The serum iron concentration of the control group was reduced by 36.12% and 28.13% than blank group, respectively ($P < 0.001$). It was notable that when compared to the saline treated control group, the serum iron level of ASP group had 1.28 and 1.14-fold increase, respectively ($P < 0.01$) (Fig. 2B, D).

ASP decreases the expression of hepcidin in vitro and in vivo

As hepcidin secreted mainly by the liver is the master regulator of systemic iron homeostasis, we analyzed whether ASP regulated hepcidin expression. The HepG2 and L-02 cells were selected and treated with 0, 0.10, 0.20 and 0.40 g/L ASP and the expression of hepcidin mRNA was analyzed by RT-PCR after 48h. Results revealed that 0.20 and 0.40 g/L of ASP decreased the expression of hepcidin in a concentration dependent manner (Fig. 3E). Next, we investigated the temporal dependence of hepcidin induction in response to 0.40 g/L ASP. The HepG2 and L-02 cells were treated for up to 96h. By means of analyzing hepcidin mRNA expression at different time points using RT-PCR, we discovered that after 48 hours administration of 0.4 g/L ASP displayed decrease of hepcidin expression in a temporal dependent manner (Fig. 3E, F). The analogical results were obtained at the protein level. Western blot analysis showed that hepcidin protein level was dramatically down-regulated in HepG2 and L-02 cells treated with ASP (Fig. 3C).

In addition, we determined the impact of ASP on hepcidin expression *in vivo*. The hepcidin protein level in liver tissues of 4T1 and H22 tumor-bearing mice was investigated by western blot. Expression of hepcidin protein in tumor bearing control group was up-regulated compared to the tumor

Fig. 3. ASP reduces hepcidin expression in vitro and in vivo. (A) Immunohistochemical analysis of livers from 4T1 and H22 tumor-bearing mice. The liver sections were subjected to IHC staining using an antibody against hepcidin. (B) The concentration of Hepcidin in serum of 4T1 and H22 tumor-bearing mice was determined by ELISA. The results are expressed as mean $\pm$ SD (** $P < 0.01$, or *** $P < 0.001$). (C) The expression level of hepcidin was analyzed by western blot in 4T1 and H22 tumor-bearing mice liver tissues. (D) Representative images of the protein level of hepcidin. Total protein was isolated from HepG2 and L-02 cells which were cultured in 0.4 g/L ASP for 96h, respectively. (E) The expression level of hepcidin gene was normalized to the corresponding level of GAPDH. Representative electrophoresis images of hepcidin and GAPDH mRNA in HepG2 and L-02 cells cultured with various concentrations of ASP for 48h. (F) Representative electrophoresis images of hepcidin and GAPDH mRNA in HepG2 and L-02 cells cultured with 0.4 g/L of ASP for various days.

free blank group, while the hepcidin protein level in ASP group was decreased significantly compared to the control group (Fig. 3D). We next used the IHC method to detect the liver expression of hepcidin *in situ*. Consistent with our western blot data, IHC results revealed that hepcidin was dramatically over-expressed in the control group compared to the blank group, whereas ASP therapies significantly decreased its abundance in both of the tumor-bearing mice (Fig. 3A).

Finally, we determined the impact of ASP on hepcidin in serum of each tumor-bearing mice by enzyme-linked immunosorbent assay (ELISA). Our results showed that hepcidin level in the control group of 4T1 and H22 tumor-bearing mice increased 1.78-fold and 1.82-fold compared with the blank group, respectively ($P < 0.01$), and compared to the control group, the levels of hepcidin in the ASP group of 4T1 and H22 tumor-bearing mice were markedly reduced by 54.55% and 47.29%, respectively ($P < 0.01$) (Fig. 3B). Taken together, our findings revealed that ASP could effectively repress the expression of hepcidin both *in vitro* and *in vivo*.

ASP regulates hepcidin expression via JAK/STAT and BMP-SMAD pathways

It is well studied that inflammation regulates hepcidin production via the IL-6/JAK2 transducer and activator of transcription JAK/STAT pathway [20]. We first determined the impact of ASP on IL-6 in serum and liver tissues of 4T1 and H22 tumor-bearing mice by ELISA and western blot. Our results showed that serum IL-6 level in the two tumor bearing control group increased 2.21-fold and 2.10-fold in comparison to that of the blank group by ELISA, respectively ($P < 0.001$), and when compared to the saline treated control group, the levels of IL-6 in the ASP group were markedly reduced by 51.09% and 33.83%, respectively ($P < 0.001$) (Fig. 4B). Consistent with ELISA data, Western blot analyses showed that the protein level of IL-6 was dramatically increased in the control group compared with the blank group, while ASP therapies significantly decreased its amount both in 4T1 and H22 tumor-bearing mice (Fig. 4A).

Previous reports indicated that JAK2, phospho-STAT3 (p-STAT3), and phospho-SMAD1/5/8 (p-SMAD1/5/8) are the key protein involved in JAK-STAT pathway and BMP-SMAD pathway regulating hepcidin production [21]. These signaling proteins are essential for hepcidin production in response to several stimulants. Therefore, the expression of these signaling molecules was examined at the protein level in liver tissues of 4T1 and H22 tumor-bearing mice. Our results showed that the expression of JAK2, p-STAT3, and p-SMAD1/5/8

Fig. 4. Signal proteins involved in hepcidin regulation are reduced after ASP treatment in vivo. (A) The expression level of IL-6, JAK2, p-STAT3, and p-SMAD1/5/8 was analyzed using western blot assays in 4T1 and H22 tumor-bearing mice liver tissues. (B) The concentration of IL-6 in serum of 4T1 and H22 tumor-bearing mice was determined by ELISA. The results are expressed as mean $\pm$ SD (* $P < 0.05$, ** $P < 0.01$, or *** $P < 0.001$).

Fig. 5. Ferritin, Tf, TfR1, and TfR2 are reduced after ASP treatment in vivo. The expression of cytokines related to iron metabolism in serum from 4T1 and H22 tumor-bearing mice was determined by ELISA. The concentration of ferritin (A), Tf (B), TfR1 (C), TfR2 (D) in 4T1 tumor-bearing mice and ferritin (E), Tf (F), TfR1 (G), TfR2 (H) in H22 tumor-bearing mice is exhibited. The results are expressed as mean $\pm$ SD (* P <0.05, ** P <0.01, or *** P <0.001).

was significantly increased in the control group compared with the blank group (Fig. 4A). Interestingly, ASP administration significantly decreased the levels of hepatic JAK2, p-STAT3, and p-SMAD1/5/8.

ASP decreases the expression of ferritin, Tf, TfR1 and TfR2 in vitro

To determine the effects of ASP on the cytokines related to iron metabolism in serum, we examined ferritin, Tf (Transferrin), TfR1 (Transferrin receptor 1), and TfR2 (Transferrin receptor 2) levels in serum of 4T1 and H22 tumor-bearing mice by ELISA. As shown in Fig. 5, serum ferritin, Tf, TfR1, and TfR2 levels in the 4T1 tumor-bearing mice control group increased 4.27-fold (P <0.001), 3.45-fold (P <0.001), 3.83-fold (P <0.001) and 1.43-fold (P <0.05) compared to tumor free blank group. And serum ferritin, Tf, TfR1, TfR2 and IL-6 levels in H22 tumor-bearing mice control group increased 3.30-fold (P <0.001), 3.37-fold (P <0.001), 3.09-fold (P <0.001) and 1.50-fold (P <0.001). However after ASP therapies, the level of these cytokines in serum were significantly reduced compared to the control group. In 4T1 tumor-bearing mice compared to the control group, the levels of serum ferritin, Tf, TfR1 and TfR2 in the ASP group were reduced by 50.73% (P <0.001), 12.20% (P >0.05), 69.36% (P <0.001) and 30.90% (P <0.01), respectively. In H22 tumor-bearing mice compared to the control group, the levels of serum ferritin, Tf, TfR1, and TfR2 in the ASP group were reduced by 34.31% (P <0.01), 18.00% (P <0.05), 39.74% (P <0.01) and 28.16% (P <0.001), respectively.

Taken together, our results demonstrated ferritin, Tf, TfR1 and TfR2 levels in serum of 4T1 and H22 bearing mice were increased, and ASP can significantly decrease these cytokines.

Discussion

As an essential nutrient element, iron is required for the growth of all cells and involved in cell metabolism, division and proliferation [22]. Cancers are often associated with disordered systemic iron homeostasis, which in turn promotes tumor development through various signaling pathways, such as iron-induced cell proliferation and oxidative stress [23, 24]. It has been shown that a low iron diet inhibits the growth of mammary adenocarcinoma

cell xenografts in rats [25, 26]. Exposure of cancer cells to iron chelators can lead to cell cycle arrest and repression of cell proliferation both *in vitro* and *in vivo* models [27, 28]. In this study we verified that iron was overloaded in tumor-bearing mice, and following treatment with ASP the iron concentration in liver, spleen, and tumor of tumor-bearing mice was significantly reduced in comparison to the control group. Meanwhile, we found that xenografted tumor growth was significantly inhibited following ASP treatment. Hepcidin secreted by liver plays a pivotal role in orchestrating iron metabolism [6]. Ferroportin is well known as export of intracellular iron [7]. Hepcidin controls systemic iron homeostasis through rapid degradation of ferroportin which increase iron retention in macrophages, hepatocytes and tumor cells, and limit the recycling of iron [7, 29]. Recent studies suggest that disordered iron homeostasis in cancers is due to abnormal regulation of hepcidin and ferroportin, both of which have prognostic significance in cancer patients [30]. In previous studies, it was found that high ferroportin and low hepcidin expression favored an extremely higher 10-year survival rate of breast cancer patients [7]_ENREF_29. Ferritin is the major storage form of iron, and it was documented that many solid tumor cells can synthesize or secrete ferritin, such as hepatic cancer, lung cancer, and breast cancer [31]. Moreover, dysregulation of ferritin is correlated with breast cancer stage and has a pejorative prognostic value in localized breast cancer [32]. In this study, our results revealed that serum hepcidin and ferritin were increased in tumor-bearing mice, and the reduction of hepcidin and ferritin synthesis was found following ASP treatment, which could robustly diminish tumor iron retention and inhibit tumor growth. Therefore, inhibition of hepcidin by ASP may prove to be a prospective approach to restrain tumor.

Almost all extracellular iron circulating in the plasma is bound to Tf, an abundant iron transport protein with high affinity for iron [33]. Cell surface TfR involving two documented types namely TfR1 and TfR2, and TfR concentration reflects iron demands of the cell. The initial event in the cellular uptake of iron is that Tf carrying iron binds to TfRs on cell surfaces, the Tf-iron-TfR complex is internalized within the cell, and then iron is released into the cell [34]. It was reported that some tumor tissues synthesize Tf for their proliferation and differentiation, such as the breast cancer cell line of MCF-7 [35, 36]. Studies also have proved that cancer cells have higher TfR1 and TfR2 expression compared to normal cells [37-39]. In present study, serum Tf, TfR1 and TfR2 levels of the control group were significantly increased compared to those of blank group. Higher expression of these iron regulatory proteins revealed a faster rate of iron absorption, which was related to an increased iron requirement by tumor cell proliferation [40]. In addition, our study also demonstrated that ASP suppressed the synthesis of these iron regulatory proteins Tf, TfR1 and TfR2, which serve as a good indicator of the intracellular status of the iron pool.

The hepcidin gene transcription in the liver is regulated by three main pathways, which include the inflammatory pathway that increases hepcidin transcription through JAK/STAT signal transduction in response to inflammatory mediators. The BMP/SMAD pathway, which mediates hepcidin upregulation by iron and hypoxia was also documented to be critical for hepcidin production, besides the erythropoietic pathway which decreases hepcidin level following an increase in the rate of erythropoiesis [11, 20, 21]. Cancer stimulates the synthesis of many inflammatory cytokines, including IL-1, IL-6 and TNF- α [41, 42]. A well characterized molecular mechanism by which inflammation regulates hepcidin production is the IL-6/JAK2 transducer and activator of transcription JAK/STAT pathway [20]. Our result suggested that IL-6, JAK2, and p-STAT3 were markedly enhanced in control group compared to blank control, and ASP could significantly down-regulate these signal proteins. Our findings indicate that ASP may mediate hepcidin by affect Janus-kinases that phosphorylate STATs. In hepcidin regulation, BMP signaling stimulate hepcidin expression by enhance the phosphorylation of SMAD1/5/8 [43]. The results presented in this study suggest that p-SMAD1/5/8 was significantly higher in tumor-bearing mice and ASP suppressed phosphorylation of SMAD1/5/8. It is speculated that the inhibition of p-STAT3 and p-SMAD1/5/8 is involved in the ASP-induced suppression of hepcidin expression. The *in vivo* model using Xenografted 4T1 and H22 cells in mice may not completely correspond

to natural growth of tumors in patients. Therefore, further experiments to elucidate the underlying mechanisms how ASP modulating hepcidin pathways in iron metabolism are required.

Acknowledgements

The authors are grateful to Prof. Yinming Liang from core facility of Immunophenotyping, Xinxiang Medical University for invaluable discussion. This work was supported by Scientific and Technological Projects of Xinxiang City [cxgg16014]; Technological Projects of Henan province [182102310259]; Medical Scientific and Technological Projects of Henan Province [201403132]; Scientific Research Fund of Xinxiang Medical University [2013QN109]; and Key scientific research projects of universities in Henan province [18A310004].

Disclosure Statement

No conflict of interests exists.

References

- Wallace DF: The Regulation of Iron Absorption and Homeostasis. Clin Biochem Rev 2016;37:51-62.
- Torti SV, Torti FM: Ironing out cancer. Cancer Res 2011;71:1511-1514.
- Richardson DR, Kalinowski DS, Lau S, Jansson PJ, Lovejoy DB: Cancer cell iron metabolism and the development of potent iron chelators as anti-tumour agents. Biochim Biophys Acta 2009;1790:702-717.
- Torti SV, Torti FM: Iron and cancer: more ore to be mined. Nature Reviews Cancer 2013;13:342-355.
- Wen CP, Lee JH, Tai YP, Wen C, Wu SB, Tsai MK, Hsieh DPH, Chiang HC, Hsiung CA, Hsu CY, Wu XF: High Serum Iron Is Associated with Increased Cancer Risk. Cancer Res 2014;74:6589-6597.
- Ganz T: Hepcidin and iron regulation, 10 years later. Blood 2011;117:4425-4433.
- Pinnix ZK, Miller LD, Wang W, D'Agostino R, Jr., Kute T, Willingham MC, Hatcher H, Tesfay L, Sui G, Di X, Torti SV, Torti FM: Ferroportin and iron regulation in breast cancer progression and prognosis. Sci Transl Med 2010;2:43ra56.
- Tesfay L, Clausen KA, Kim JW, Hegde P, Wang X, Miller LD, Deng Z, Blanchette N, Arvedson T, Miranti CK, Babitt JL, Lin HY, Peehl DM, Torti FM, Torti SV: Hepcidin regulation in prostate and its disruption in prostate cancer. Cancer Res 2015;75:2254-2263.
- Marques O, Porto G, Rema A, Faria F, Cruz Paula A, Gomez-Lazaro M, Silva P, Martins da Silva B, Lopes C: Local iron homeostasis in the breast ductal carcinoma microenvironment. BMC Cancer 2016;16:187.
- Kamai T, Tomosugi N, Abe H, Arai K, Yoshida K: Increased serum hepcidin-25 level and increased tumor expression of hepcidin mRNA are associated with metastasis of renal cell carcinoma. BMC Cancer 2009;9:270.
- Reichert CO, da Cunha J, Levy D, Maselli LMF, Bydlowski SP, Spada C: Hepcidin: Homeostasis and Diseases Related to Iron Metabolism. Acta Haematologica 2017;137:220-236.
- Ganz T: Cellular iron: ferroportin is the only way out. Cell Metab 2005;1:155-157.
- Qiao B, Sugianto P, Fung E, Del-Castillo-Rueda A, Moran-Jimenez MJ, Ganz T, Nemeth E: Hepcidin-induced endocytosis of ferroportin is dependent on ferroportin ubiquitination. Cell Metab 2012;15:918-924.
- Chen XP, Li W, Xiao XF, Zhang LL, Liu CX: Phytochemical and pharmacological studies on Radix Angelica sinensis. Chin J Nat Med 2013;11:577-587.
- Jin M, Zhao K, Huang Q, Xu C, Shang P: Isolation, structure and bioactivities of the polysaccharides from Angelica sinensis (Oliv.) Diels: a review. Carbohydr Polym 2012;89:713-722.
- Zhang Y, Zhou T, Wang H, Cui Z, Cheng F, Wang KP: Structural characterization and *in vitro* antitumor activity of an acidic polysaccharide from Angelica sinensis (Oliv.) Diels. Carbohydr Polym 2016;147:401-408.

- 17 Cheng Y, Zhou J, Li Q, Liu Y, Wang K, Zhang Y: The effects of polysaccharides from the root of *Angelica sinensis* on tumor growth and iron metabolism in H22-bearing mice. *Food Funct* 2016;7:1033-1039.
- 18 Xia XY, Peng RX, Kong R, Yang ZQ, Chen X: [Effects of *Angelica sinensis* polysaccharides on hepatic drug metabolism enzymes activities in mice]. *Zhongguo Zhong Yao Za Zhi* 2003;28:149-152.
- 19 Ren F, Qian XH, Qian XL: Astragalus polysaccharide upregulates hepcidin and reduces iron overload in mice via activation of p38 mitogen-activated protein kinase. *Biochem Biophys Res Commun* 2016;472:163-168.
- 20 Stoian I, Manolescu B, Atanasiu V, Lupescu O, Busu C: IL-6 - STAT-3 - hepcidin: linking inflammation to the iron metabolism. *Rom J Intern Med* 2007;45:305-309.
- 21 Darshan D, Anderson GJ: Interacting signals in the control of hepcidin expression. *Biometals* 2009;22:77-87.
- 22 Wang J, Pantopoulos K: Regulation of cellular iron metabolism. *Biochem J* 2011;434:365-381.
- 23 Fonseca-Nunes A, Jakszyn P, Agudo A: Iron and cancer risk--a systematic review and meta-analysis of the epidemiological evidence. *Cancer Epidemiol Biomarkers Prev* 2014;23:12-31.
- 24 Garmendia CA, de Los Reyes M, Madhivanan P: Systematic review and meta-analysis on iron and cancer risk-letter. *Cancer Epidemiol Biomarkers Prev* 2014;23:1435.
- 25 Chen Y, Zhang S, Wang X, Guo W, Wang L, Zhang D, Yuan L, Zhang Z, Xu Y, Liu S: Disordered signaling governing ferroportin transcription favors breast cancer growth. *Cell Signal* 2015;27:168-176.
- 26 Manz DH, Blanchette NL, Paul BT, Torti FM, Torti SV: Iron and cancer: recent insights. *Ann N Y Acad Sci* 2016;1368:149-161.
- 27 Bystrom LM, Rivella S: Cancer cells with irons in the fire. *Free Radic Biol Med* 2015;79:337-342.
- 28 Pangjit K, Tantiphaipunwong P, Sajjapong W, Srichairatanakool S: Iron-chelating, free radical scavenging and anti-proliferative activities of *Azadirachta indica*. *J Med Assoc Thai* 2014;97:S36-43.
- 29 Lamy PJ, Durigova A, Jacot W: Iron homeostasis and anemia markers in early breast cancer. *Clin Chim Acta* 2014;434:34-40.
- 30 Zhang S, Chen Y, Guo W, Yuan L, Zhang D, Xu Y, Nemeth E, Ganz T, Liu S: Disordered hepcidin-ferroportin signaling promotes breast cancer growth. *Cell Signal* 2014;26:2539-2550.
- 31 Alkhateeb AA, Connor JR: The significance of ferritin in cancer: anti-oxidation, inflammation and tumorigenesis. *Biochim Biophys Acta* 2013;1836:245-254.
- 32 Shpyleva SI, Tryndyak VP, Kovalchuk O, Starlard-Davenport A, Chekhun VF, Beland FA, Pogribny IP: Role of ferritin alterations in human breast cancer cells. *Breast Cancer Res Treat* 2011;126:63-71.
- 33 Szoke D, Panteghini M: Diagnostic value of transferrin. *Clin Chim Acta* 2012;413:1184-1189.
- 34 Kawabata H, Sakamoto S, Masuda T, Uchiyama T, Ohmori K, Koeffler HP, Takaori-Kondo A: Roles of transferrin receptors in erythropoiesis. *Rinsho Ketsueki* 2016;57:951-958.
- 35 Park S, Yoon SY, Kim KE, Lee HR, Hur DY, Song H, Kim D, Bang SI, Cho DH: Interleukin-18 induces transferrin expression in breast cancer cell line MCF-7. *Cancer Lett* 2009;286:189-195.
- 36 Wang SJ, Gao C, Chen BA: Advancement of the study on iron metabolism and regulation in tumor cells. *Chin J Cancer* 2010;29:451-455.
- 37 Walker RA, Day SJ: Transferrin receptor expression in non-malignant and malignant human breast tissue. *J Pathol* 1986;148:217-224.
- 38 Soyer HP, Smolle J, Torne R, Kerl H: Transferrin receptor expression in normal skin and in various cutaneous tumors. *J Cutan Pathol* 1987;14:1-5.
- 39 Tortorella S, Karagiannis TC: Transferrin receptor-mediated endocytosis: a useful target for cancer therapy. *J Membr Biol* 2014;247:291-307.
- 40 Torti SV, Torti FM: Iron and cancer: more ore to be mined. *Nat Rev Cancer* 2013;13:342-355.
- 41 Vendramini-Costa DB, Carvalho JE: Molecular link mechanisms between inflammation and cancer. *Curr Pharm Des* 2012;18:3831-3852.
- 42 Yang L, Han S, Sun Y: An IL6-STAT3 loop mediates resistance to PI3K inhibitors by inducing epithelial-mesenchymal transition and cancer stem cell expansion in human breast cancer cells. *Biochem Biophys Res Commun* 2014;453:582-587.
- 43 Kanamori Y, Murakami M, Matsui T, Funaba M: The regulation of hepcidin expression by serum treatment: requirements of the BMP response element and STAT- and AP-1-binding sites. *Gene* 2014;551:119-126.