JBUON 2017; 22(5): 1328-1332
ISSN: 1107-0625, online ISSN: 2241-6293 • www.jbuon.com
E-mail: editorial_office@jbuon.com

ORIGINAL ARTICLE

Hepcidin and iron metabolism in the pathogenesis of prostate cancer

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Summary

Purpose: *To investigate the relationship between Hepcidin and iron metabolism, and cell proliferation, migration, and apoptosis in prostate cancer cells.*

Methods: *PC3 prostate cancer cells were cultured in vitro and divided into the control group, Hepcidin overexpression group, and Hepcidin low expression group. Prostate specific antigen (PSA) and soluble transferrin receptor (sTfR) levels were measured by ELISA. The levels of the Hepcidin receptor membrane transporter, Ferroportin, were determined by Western blot. The intracellular iron distribution was determined by immunofluorescence assay. The cell proliferation rate was determined by MTT assay. Cell migration was measured by wound healing assay. Apoptosis was measured by flow cytometry.*

Results: *Compared with the control group, higher PSA level ($p<0.05$), lower sTfR level ($p<0.05$), lower Ferroportin level ($p<0.05$), lower intracellular iron level ($p<0.05$), higher cell proliferation and migration rate, and lower apoptotic rate ($p<0.05$) were found in the Hepcidin overexpression group. The opposite results were found in the Hepcidin low expression group.*

Conclusions: *Hepcidin is highly expressed in prostate cancer cells, and can regulate cell proliferation, migration, and apoptosis by increasing intracellular iron transportation.*

Key words: *apoptosis , ferroportin, hepcidin, migration, proliferation, prostate cancer*

Introduction

Prostate cancer is a malignancy with high morbidity and mortality in males [1]. Previous studies [2,3] found that intracellular iron overload is important for the proliferation, metastasis, and angiogenesis of prostate cancer cells. Hepcidin is a core molecule involved in iron metabolism. Hepcidin gene knockout in mice can lead to iron overload [4], while Hepcidin overexpression in mice and humans can cause iron deficiency anemia [5]. Hepcidin can bind its receptor, Ferroportin, to transfer intracellular free iron. Low expression of Ferroportin can increase tumor cell invasion [6-7]. Based on this observation, the aim of this study was to analyze the relationship between Hepcidin and iron metabolism, and cell proliferation, migration and apoptosis in prostate cancer cells.

Methods

Cell culture

PC3 human prostate cancer cells were purchased from the Shanghai Institutes for Biological Sciences of the Chinese Academy of Sciences. Routine cell resuscitation was performed. Cells were then cultured in MEM complete medium containing 10% FBS in an

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Received: 11/03/2017; Accepted: 25/03/2017

incubator (37°C, 5% CO_2). The medium was replaced, and cells were divided among different culture bottles on the following day. Finally, the cells were collected and resuspended, and the cell density was adjusted to 2×10^6/ml.

Experimental grouping

The cells were transfected at the logarithmic growth phase. There were three groups of cells: the control group, Hepcidin overexpression group, and Hepcidin low expression group. Transfection was performed using Lipofectamine™ reagent (Sigma, St. Louis, MO, USA) according to the manufacturer's instructions. Hepcidin overexpression and low expression were detected via real-time PCR using SYBR Green Master Mix (Applied Biosystems, Foster City, California, USA). The primers used for Hepcidin partial sequence (175 bp) amplification were designed according to Gene Bank and synthesized by Sangon Biotech (Shanghai, China). The primers used for PCR amplification were (sense) 5'-GACCAGTGGCTCTGTTTTCC-3', and (anti-sense) 5'-CACATCCCACACTTTGATCG-3'. The cells were cultured for an additional 24 hrs after PCR validation.

Indicator detection

PSA and sTfR levels were measured by ELISA. The levels of the Hepcidin receptor membrane transporter, Ferroportin, were measured by Western blot. Intracellular iron distribution was determined by immunofluorescence assay. The cell proliferation rate was determined by MTT assay. Cell migration was measured by wound healing assay. Cell apoptosis was measured by flow cytometry.

ELISA

Cell culture medium was centrifuged (2000 g) for 20 min, and the supernatant was collected. PSA and sTfR reagents were purchased from Beyotime Biotechnology (Jiangsu, China). The microplate reader was purchased from Invitrogen (Carlsbad, California, USA). All experiments were performed in triplicate according to the instructions, and the values were averaged.

Western blot

RIPA Buffer (Sigma, St. Louis, MO, USA) was added to each group of cells for extraction of total protein. The Coomassie blue method was applied for quantification, and a β-actin (endogenous control) antibody was used for standardization. Protein (30 μg) was subjected to 8% SDS-PAGE followed by transfer to PVDF membranes. The primary monoclonal antibodies (mouse anti-human) against Ferroportin and β-actin (1: 2000, Sigma, St. Louis, MO, USA) were added and incubated with the membranes overnight. Next, rabbit anti-mouse polyclonal secondary antibody (1: 500, Sigma, St. Louis, MO, USA) was added at room temperature for 4 hrs, followed by washing with PBS, and color development with ECL solution. The results were scanned. Gel imaging software (Invitrogen, Carlsbad, California, USA) was used for semi-quantitative analysis.

Immunofluorescence assay

CA-AM (0.25 nM) (R&D, Minneapolis, MN, USA) was added to 1 ml of cell suspension, and incubated at 37°C for 5 min. Cells were then washed three times with 20 mM Hepes buffer (Santa Cruz, CA, USA) containing 1 mg/ml BSA, for 15 min each time. The cells were resuspended in 4 ml of HBSS buffer (GE, Fairfield, Connecticut, USA), and the fluorescence value (A) was measured by a microplate reader. Next, l00 mM iron chelate BIP (Bio-Rad, Hercules, California, USA) was added and incubated at 37°C for 30 min. Cells were then washed with HBSS buffer, resuspended, and the fluorescence value (B) was measured. The intracellular iron content = B value − A value.

MTT assay

Cells were seeded in 96-well plates, with 200 μl of cell suspension per well. Each group was analyzed in triplicate. Next, 20 μl (5 mg/ml) of MTT (Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China) were added to each well, and the cells were cultured for an additional 4 hrs followed by centrifugation (2000 g) for 10 min. The supernatant was discarded and 200 μl of DMSO (Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China) were added to each well. The mixture was oscillated to ensure that crystals were completely dissolved. The optical density (OD) value of each well was measured by a microplate reader at 490 nm and 630 nm, and the rate of inhibition of cell proliferation was calculated.

Wound healing assay

Cells were seeded in 6-well plates, with 2 ml of culture medium in each well. A 10 μl tip was used to scratch a straight line through the cell monolayer. Next, medium containing 1% serum was added, and pictures were acquired at 0 h and 24 hrs. The migration distance was measured. The experiments were performed in triplicate and the average values were calculated.

Flow cytometry

The cells in each group were centrifuged at 3000 g for 15 min, PBS was used to wash the precipitated cells, and the supernatant was discarded. Next, 500 μl of Binding Buffer (BD, Lake Franklin, New Jersey state, USA) were added to the cell suspensions, 5 μl of Annexin V-FITC (BD) and 5 μl of propidium iodide (PI) were added and mixed in turn at room temperature, and the mixture was left to stand in the dark for 15 min. Detection was then performed after 1 hr.

Statistics

Statistical analysis was performed using SPSS20.0 software (SPSS Inc., Chicago, IL, USA). Data are presented as mean±standard deviation. One-way ANOVA analysis was used for intragroup comparisons. Intergroup comparisons were performed by the LSD-t method and a value <0.05 was considered statistically significant.

Results

PSA and sTfR expression levels

Compared with the control group, higher PSA levels and lower sTfR levels were found in the Hepcidin overexpression group ($p<0.05$), while the opposite results were found in the Hepcidin low expression group (Table 1).

Table 1. Expression levels of PSA and sTfR

Groups	*PSA Mean±SD*	*sTfR Mean±SD*
Control group	24.5±5.6	6.8±2.2
Hepcidin overexpression group	28.2±6.3	6.2±2.4
Hepcidin low expression group	20.3±4.8	7.4±2.6
F	6.639	6.231
p	0.008	0.012

For abbreviations see text

Ferroportin expression levels

Compared with the control group, the levels of Ferroportin were decreased in the Hepcidin overexpression group, while they were increased in the Hepcidin low expression group ($p<0.05$) (Figure 1).

Intracellular iron distribution

Compared with the control group, intracellular iron levels were increased in the Hepcidin overexpression group and decreased in the Hepcidin low expression group ($p<0.05$) (Figure 2).

Cell proliferation rate, migration distance and apoptosis rate

Compared with the control group, the cell proliferation rate and migration distance in the Hepcidin overexpression group were increased, and the apoptotic rate was decreased ($p<0.05$). The opposite results were found in the Hepcidin low expression group (Table 2).

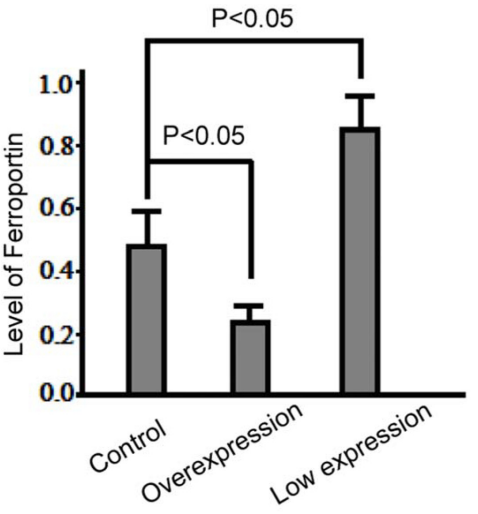

Figure 1. Ferroportin levels as determined by Western blot. The levels of Ferroportin were significantly lower in the Hepcidin overexpression group compared with the control group, while the levels of Ferroportin were significantly increased in the Hepcidin low expression group compared with the control group.

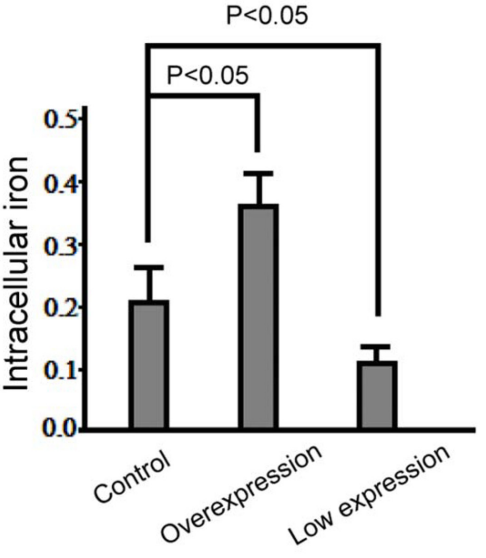

Figure 2. Intracellular iron distribution as determined by immunofluorescence assay. The intracellular iron levels in the Hepcidin overexpression group were significantly higher than in the control group, while the intracellular iron levels in the Hepcidin low expression group were significantly lower than in the control group.

Table 2. Cell proliferation rate, migration distance and apoptosis rate

Groups	*Proliferation rate % Mean±SD*	*Migration distance, μm, Mean±SD*	*Apoptosis rate % Mean±SD*
Control group	75.6±12.3	856.9±124.5	35.4±8.9
Hepcidin overexpression group	92.3±16.7	1124.7±168.9	16.9±7.6
Hepcidin low expression group	66.8±10.5	648.2±124.7	54.7±13.2
F	10.235	12.455	9.628
p	0.000	0.000	0.000

SD: standard deviation

Discussion

Compared with the control group, higher PSA level, lower sTfR level, lower Ferroportin level, lower intracellular iron level, higher cell proliferation and migration rate, and lower apoptotic rate were found in the Hepcidin overexpression group. The opposite results were found in the Hepcidin low expression group. Therefore, we believe that Hepcidin expression in prostate cancer cells can both increase intracellular iron transportation and regulate cell proliferation, migration and apoptosis.

Iron is an essential trace element in humans that not only maintains normal physiological function, but also maintains tumor cell growth. Regulating iron metabolism and reducing the use of intracellular iron are expected to represent new directions in anti-tumor therapy [8]. Hepcidin, which is synthesized and secreted by hepatocytes, can reduce the release of intracellular free iron from reticuloendothelial cells, reduce iron absorption of the duodenum, and regulate the balance of iron metabolism [9]. The Hepcidin gene is located on human chromosome 19, with two stable beta-folded structures. Infection, injury, and inflammation can significantly increase the expression of Hepcidin [10,11]. A study has shown that IL-6 treatment can significantly increase Hepcidin mRNA expression in hepatocytes [12]. The Hepcidin promoter contains an IL-6 responsive element, and IL-6/STAT3 signaling plays an important role in the regulation of high expression of Hepcidin [13]. PSA level is a prostate cancer-specific tumor indicator, and sTfR level is an indicator of intracellular iron content and iron storage. Abnormal expression of Hepcidin can affect PSA and sTfR expression levels in prostate cancer cells, thereby affecting the growth of tumor cells [14,15].

As the only iron output protein in humans, Ferroportin is widely distributed in the small intestine, liver, spleen, and other organs. Moreover, Ferroportin is mainly regulated by Hepcidin [16]. A study has shown that Ferroportin can serve as an indicator in the diagnosis and prognosis of breast cancer [17]. Hepcidin can form a complex with Ferroportin to induce Ferroportin degradation in the cytoplasm of cells, leading to decreased Ferroportin content in cell membranes, to block the iron output of intestinal mucosal cells, which in turn increases the intracellular iron content [18]. The occurrence and progression of tumors increases the need for intracellular iron. Prostate cancer bone metastases are also closely related to the abnormal expression of Hepcidin [19]. The use of RNA interference technology also confirmed that Hepcidin expression is related to prostate cancer cell proliferation and apoptosis [20].

A key finding of this study was that the occurrence of prostate cancer is associated with Hepcidin and abnormal iron metabolism, which may represent a new basis for the diagnosis and treatment of tumors. A limitation of this study was that the optimal level of Hepcidin expression has not been identified. Furthermore, the studies on the overexpression and low expression of Hepcidin were performed in prostate cancer cells, while normal control cells were not used. Experiments in animal models and *in vivo* clinical studies are required to further determine the relationship between Hepcidin and iron metabolism and tumorigenesis.

Conflict of interests

The authors declare no confict of interests.

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