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Disordered hepcidin–ferroportin signaling promotes breast cancer growth

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ABSTRACT

Iron homeostasis is strictly governed in mammals; however, disordered iron metabolism (such as excess iron burden) is recognized as a risk factor for various types of diseases including cancers. Burgeoning evidence indicates that the central signaling of iron homeostasis, the hepcidin–ferroportin axis, is misregulated in cancers. Nonetheless, the mechanisms of misregulated expression of iron-related genes along this signaling in cancers remain largely unknown. In the current study, we found increased levels of serum hepcidin in breast cancer patients. Reduction of hepatic hepcidin through administration of heparin restrained tumorigenic properties of breast tumor cells. Mechanistic investigation revealed that increased iron, bone morphogenetic protein-6 (BMP6) and interleukin-6 (IL-6) jointly promoted the synthesis of hepatic hepcidin. Tumor hepcidin expression was marginally increased in breast tumors relative to adjacent tissues. In contrast, tumor ferroportin concentration was greatly reduced in breast tumors, especially in malignant tumors, compared to adjacent tissues. Elevation of ferroportin concentration inhibited cell proliferation in vitro and in vivo by knocking down tumor hepcidin expression. Additionally, increased IL-6 was demonstrated to jointly enhance the tumorigenic effects of iron through enforcing cell growth. Our combined data overall deciphered the machinery that altered the hepcidin–ferroportin signaling in breast cancers. Thus, targeting the hepcidin–ferroportin signaling would represent a promising therapeutics to restrain breast cancer growth.

1. Introduction

Mammalian cells have a fine-tuned regulatory system to maintain iron balance. However, misregulation of this system would elicit detrimental effects on cells, such as the oncogenic effect of intracellularly retained free iron [1,2]. Deregulated systemic iron homeostasis contributes to cancer risk and progression [2,3]. The contributory role of iron in cancers could be mediated by: a) overproduction of ROS and free radicals through iron-dependent Fenton reaction; b) induction of oxidative stress-responsive transcriptional factors and pro-inflammatory cytokines; c) iron-mediated hypoxia signaling; and d) promotion of DNA synthesis driven by iron-containing ribonucleotide reductase [4].

Although studies of iron have been ongoing for a long time, the molecular bases of systemic iron homeostasis were only recently identified with the characterization of the hepcidin–ferroportin axis [5,6]. Hepcidin, a peptide hormone, is prominently synthesized by the liver, and secreted into serum followed by tissue localization through circulation [7]. Hepcidin inhibits iron absorption from the duodenum and iron egress from macrophages and hepatocytes through its binding and inducing degradation of iron exporter ferroportin [8]. Deregulated hepcidin–ferroportin signaling is implicated not only in iron diseases, e.g. hereditary hemochromatosis and anemia of inflammation [5,9,10], but also in cancers [11–13]. Previous studies have revealed that increased serum hepcidin accompanies multiple cancers, such as myeloma, renal cell carcinoma and prostate cancer [11,12,14,15]; however, the effects of abnormal circulating hepcidin on tumor behaviors remain unexploited. Moreover, recent studies suggest that iron metabolism in cancer cells themselves is also adapted for the enhanced demand of iron necessary for rapid growth of cancer cells [2]. Tumor cells were also noted to express hepcidin and its receptor

Abbreviations: FAC, Ferric Ammonium Citrate; DCF-DA, dichlorofluorescein-diacetate; PI, propidium iodide; IO, iron-overloaded; MFPs, mammary fat pads; IP, intraperitoneal injection; CA-AM, calcein acetoxymethyl ester; FACS, Fluorescence Activated Cell Sorting; H&E, hematoxylin and eosin; Abs, antibodies; NAC, N-acetyl-cysteine; ROS, reactive oxygen species; ID, iron-deficient; IV, intravenous injection; PBS, phosphate-buffered saline; DFO, Deferoxamine; DAB, diaminobenzidine.

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ferroportin [15,16]. Moreover, reduced ferroportin expression correlated with poor prognosis, with a better survival rate for patients with high ferroportin levels in cancer patients [13,17]. Nonetheless, studies of the adaptive metabolism for tumor iron that facilitates cell growth are still rare, and the molecular mechanisms responsible for abnormal regulation of hepcidin/ferroportin in tumor cells remain elusive. In this study, we aim to elucidate the molecular bases underlying the misregulated hepcidin–ferroportin signaling in breast cancers. Our combined data highlight an important role of the disordered hepcidin–ferroportin signaling in promoting breast cancer growth, and provide a rationale for targeting this signaling for the therapeutics of breast cancers.

2. Materials and methods

2.1. Cell culture

Human breast cancer cell lines MDA-MB-231, MCF-7, T47D and SK-BR-3, mouse breast cancer cell line 4T1, and human hepatic carcinoma cell lines SMMC-7721 and HepG2 and hepatic epithelial cell line L-02 were obtained from the Shanghai Cell Bank of Type Culture Collection of Chinese Academy of Sciences. Cells were cultured in RPMI 1640 medium (Gibco), supplemented with 10% fetal bovine serum (FBS, Hyclone) and 100 U/ml penicillin–streptomycin (Hyclone) at 37 °C under 5% CO_2.

2.2. Clinical specimens

All sera and tumor specimens were obtained from Weihai Wendeng Central Hospital, China, with written consent in accordance with the regulations of the Institutional Ethics Committee. Overall, 155 sporadic breast cancer patients were enlisted with an average age of 47.25 ± 0.74 years old, and breast cancers were diagnosed based on surgical and pathological characteristics. Blood samples were collected prior to any therapeutics and surgery. Tumor specimens were obtained from the initial surgery for all patients. Healthy controls were age-matched healthy women (48.07 ± 0.98 years old) who were selected from the same local district and had no history of tumors, systemic disorders, liver diseases, and inflammatory diseases. Healthy individuals provided informed consent for anonymous use of their blood for scientific research. All fresh specimens were immediately frozen under −80 °C for future analyses.

2.3. Animal experiments

All animal care and surgical procedures were approved by the Animal Ethics Committee at the Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences. All mice were purchased from the Vital River Laboratories, China and housed under a sterile and pathogen-free environment. Human breast cancer MDA-MB-231 cells and mouse breast cancer 4T1 cells were used to study in vivo tumorigenesis and tumor growth. The experimental setup for cancer cell inoculation and tumor graft implantation was similar to the methods described previously [18,19]. Tumor growth was monitored every 2 days by measuring the tumor size with a vernier caliper by a blinded observer and calculating according to the formula $\pi/6 \times L \times W^2$. Mice were sacrificed when tumors reached a size of 1.0 cm^3 or caused significant morbidity to animals. Tumorigenic rate was calculated by dividing the number of tumor-bearing mice by the number of total mice in each group.

2.4. Mouse models of iron overload and iron deficiency

Iron overload was induced through intraperitoneal injection (IP) of iron-dextran (Sigma-Aldrich) into Balb/c mice at 250 mg/kg body weight twice a week for 4 weeks, similar to a previous study [20]. To induce iron deficiency, Balb/c mice were put on an iron-deficient diet for 4 weeks as previously described [21].

2.5. RNA extraction and qRT-PCR analysis

Total RNAs were isolated from cells using TRIzol (Invitrogen) according to the manufacturer's instructions. Tissue samples were first pulverized in liquid nitrogen and then total RNAs were extracted with TriPure (Roche) following the instruction provided by the manufacturer. Quantitative measurements of gene expression were carried out with SYBR Green qPCR master mix (Qiagen) on qPCR Systems Mx3005P (Stratagene). GAPDH or HPRT1 was used as an internal control for human or mouse samples, respectively. Primer sequences are shown in Table 1.

2.6. Western blotting and ELISA assay

Cultured cells after treatments were harvested and washed twice with phosphate-buffered saline (PBS). Total proteins were extracted with ice-cold RIPA lysis buffer (Solarbio, China) containing protease inhibitor cocktail (Roche). Solid tissues were snap-frozen in liquid nitrogen and homogenized in the abovementioned lysis buffer. Equal amounts of protein lysates (30–50 μg/sample) were subject to 8–12% SDS-PAGE and Western blot analysis as described previously [22]. Antibodies were anti-GAPDH (1:1000, Santa Cruz Biotechnology), ferritin light chain antibody (1:500, Abcam) and ferroportin antibody (1:500, Sigma-Aldrich).

The concentrations of BMP6, BMP4, IL-6 and hepcidin were measured in sera from breast cancer patients and healthy individuals using ELISA kits according to the manufacturer's instructions (BMP6 and BMP4 kits from RayBiotech, IL-6 kit from R&D and hepcidin kit from DRG).

2.7. Determination of iron content, intracellular LIP measurement and iron staining

Serum iron content was determined with a kit following the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, China). Tissue non-heme iron was assayed as previously described [23]. The intracellular LIP level was determined according to an established method [24]. Briefly, cells were collected and washed twice with PBS, followed by incubation with 0.5 μM CA-AM (calcein acetoxymethyl ester) (Sigma-Aldrich) for 15 min at 37 °C. Thereafter, cells were washed twice with PBS and were then equally divided into two parts with one treated with 100 μM DFO (Sigma-Aldrich) for 1 h at 37 °C and the other left untreated. Calcein was excited at 488 nm and measured at 525 nm with a flow cytometer (BD, FACSCalibur). The intracellular LIP was calculated after deduction of the cellular fluorescence in DFO-treated cells by that in the untreated cells.

Table 1
Oligonucleotide primers for qRT-PCR.

Name	Sequence (5′ → 3′)
hsa_hepcidin sense	CCTGACCAGTGGCTCTGTTT
hsa_hepcidin anti-sense	CACATCCCACACTTTGATCG
hsa_ferroportin sense	ACCTCGCTGGTGGTACAGAATGTT
hsa_ferroportin anti-sense	AGCAGGAAGTGAGAACCCATCCAT
hsa_GAPDH sense	GAAGGTGAAGGTCGGAGT
hsa_GAPDH anti-sense	GAAGATGGTGATGGGATTTC
mmu_hepcidin sense	CTGAGCAGCACCACCTATCTC
mmu_hepcidin anti-sense	TGGCTCTAGGCTATGTTTTGC
mmu_BMP6 sense	AACGCCCTGTCCAATGACG
mmu_BMP6 anti-sense	ACTCTTGCGGTTCAAGGAGTG
mmu_IL-6 sense	CTGCAAGAGACTTCCATCCAG
mmu_IL-6 anti-sense	AGTGGTATAGACAGGTCTGTTGG
mmu_HPRT1 sense	GCTTGCTGGTGAAAAGGACCTCTCGAAG
mmu_HPRT1 anti-sense	CCCTGAAGTACTCATTATAGTCAAGGGCAT

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