Ferritin: A potential serum marker for lymph node metastasis in head and neck squamous cell carcinoma

ZHANGWEI HU1*, LEI WANG1*, YONG HAN2, FEN LI3, ANYUAN ZHENG1, YONG XU1, FEI WANG1, BOKUI XIAO1,3, CHEN CHEN3 and ZEZHANG TAO1,3

1Department of Otolaryngology Head and Neck Surgery, Renmin Hospital of Wuhan University, Wuhan, Hubei 430060; 2Clinical Research Institute, Zhejiang Provincial People's Hospital, Hangzhou, Zhejiang 310014; 3Research Institute of Otolaryngology Head and Neck Surgery, Renmin Hospital of Wuhan University, Wuhan, Hubei 430060, P.R. China

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Correspondence to: Dr Zezhang Tao, Department of Otolaryngology Head and Neck Surgery, Renmin Hospital of Wuhan University, 99 Zhangzhidong Street, Wuhan, Hubei 430060, P.R. China E-mail: taozezhang@hotmail.com

Dr Chen Chen, Research Institute of Otolaryngology Head and Neck Surgery, Renmin Hospital of Wuhan University, 99 Zhangzhidong Street, Wuhan, Hubei 430060, P.R. China E-mail: chenchen_md@whu.edu.cn

*Contributed equally

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Abstract. Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer in the world (1). A total of ~600,000 new cases are reported every year, with a marked proportion of these cases in China (2). HNSCC frequently occurs as a heterogeneous tumor with an aggressive phenotype (3). In clinical practice, metastasis, particularly cervical lymph node metastasis, frequently occurs during the progression of the disease (4). Regional cervical lymph node metastasis is closely associated with the poor prognosis of patients with HNSCC (5). Current treatments for HNSCC have limited success in improving patient prognosis. Therefore, identifying potential novel markers may be a promising strategy to treat HNSCC.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer in the world (1). A total of ~600,000 new cases are reported every year, with a marked proportion of these cases in China (2). HNSCC frequently occurs as a heterogeneous tumor with an aggressive phenotype (3). In clinical practice, metastasis, particularly cervical lymph node metastasis, frequently occurs during the progression of the disease (4). Regional cervical lymph node metastasis is closely associated with the poor prognosis of patients with HNSCC (5). Current treatments for HNSCC have limited success in improving patient prognosis. Therefore, identifying potential novel markers may be a promising strategy to treat HNSCC.

Iron metabolism is associated with tumor growth and promotes tumor cell proliferation in HNSCC (6,7). The major storage form of iron in the human body is ferritin (8). Ferritin has been studied for nearly 80 years. However, it remains a key molecule, with novel characteristics continuing to be discovered. Recent studies revealed that the expression levels of ferritin were closely associated with a number of malignant tumors, including lung cancer (9) and primary hepatocellular carcinoma (10). However, the role of ferritin in HNSCC tumorigenesis and development is not fully understood.

In the present study, serological and histological experiments, in addition to Gene Expression Omnibus (GEO) datasets, were used to investigate the expression levels of ferritin in HNSCC. The primary aim was to clarify whether ferritin may serve as a biomarker for HNSCC diagnosis and metastasis prediction.

Materials and methods

Patient selection for serum ferritin (SF) detection. As presented in Table I, 281 patients, including 44 patients with mucosal
inflammation (nasopharyngeal epithelium or throat mucous membrane), 133 with benign tumors (vocal polyps, n=108; cyst of epiglottis, n=25), 20 with precancerosis (vocal leukoplakia, n=1; atypical hyperplasia, n=19) and 84 with cancer (carcinoma in situ, n=14; HNSCC without metastasis, n=40; HNSCC with metastasis, n=30), were selected for the study. The inclusion criteria were: i) patient diagnoses were based on conventional clinical, radiographic and histopathological or cytological criteria; ii) patients had previously undergone primary tumor resection. The 70 patients with HNSCC (not including carcinoma in situ) additionally underwent appropriate cervical lymph node dissection at Renmin Hospital of Wuhan University (Wuhan, China) between January 2013 and January 2015. The exclusion criteria were: i) patients with severe systemic disorders (such as diabetes mellitus, renal or heart failure); ii) patients with diseases that affect SF levels (such as liver injury, precancerous anemia or other types of cancer); iii) patients who underwent previous chemotherapy or radiotherapy.

 Serum sample collection and analyses. Venous blood samples (3-5 ml) were collected in a plain vial at diagnosis, prior to surgery. The samples were stored at room temperature for 2 h. Serum samples were obtained following centrifugation at 1,500 x g at 4°C for 5 min and immediately stored at -70°C until use. The expression levels of SF were detected using the chemiluminescent immunoassay method with a Siemens Centaur XP fully-automated chemiluminescence immunoassay analyzer (Siemens Healthcare GmbH, Erlangen, Germany) in the Department of Clinical Laboratory, Renmin Hospital of Wuhan University, and the correlate Quantitative Assay Kit for ferritin (cat. no. 012245) was purchased from Siemens Healthcare GmbH.

 Ethics statement. The present study was approved by the ethics committee of Renmin Hospital of Wuhan University. All specimens were collected from patients who provided written informed consent, in accordance with the principles of the Declaration of Helsinki.

 Patient selection for iron staining and immunohistochemical staining. A total of 70 sets of paraffin-embedded specimens were obtained from the pathology department, including 40 patients without and 30 with cervical node metastasis. Details are presented in Table II. Each set contained one primary tumor tissue and one corresponding cervical lymph node tissue. The tissues were fixed in 4% formaldehyde for 24 h at 4°C, and were routinely processed into paraffin blocks. Each tissue was divided into at least three paraffin-embedded sections: One for detecting iron content using iron staining; and the remaining two for detecting ferritin H (FTH) and ferritin L (FTL), respectively, using immunohistochemistry (IHC).

 Neck color Doppler ultrasonography. The neck lymph nodes of the 70 patients with HNSCC were detected using color Doppler ultrasonography prior to surgery, and the results were obtained from the radiology department. The features of the nodes included size (longitudinal diameter, LD) and shape (long axis/short axis or L/S ratio). For size, the dividing line was 1 cm LD, and for the shape, 2x L/S ratio.

 Tissue microarray for IHC. Two tissue microarrays (TMAs) (no. HN803b) with the same patients’ information were purchased from US Biomax, Inc. (Rockville, MD, USA) to examine differences in the expression of FTH and FTL between HNSCC and normal tissues. Each TMA consisted of 11 normal and 69 HNSCC tissue samples, with the mean age of patients being 53.4 years (range 15-90 years). Details of the TMA characteristics are presented in Table III.

 Iron staining. Iron staining (also termed Prussian blue staining) was performed using a 5% potassium hexacyanoferrate trihydrate and hydrochloric acid solution. Staining incubation time was ~30 min at room temperature. Subsequently, the sections were rinsed with water, counterstained with nuclear fast red, dehydrated, and covered.

 IHC staining. The 3-5 µm sections were deparaffinized with standard pure xylene for 15 min, three times at room temperature and hydrated in an alcohol gradient. PBS was used to wash the sections. Antigen retrieval was performed in boiling citrate buffer (pH 6.0) for 15 min. The sections were dried down to room temperature in the citrate buffer. Following washing of the sections with PBS three times for 5 min, 0.3% hydrogen peroxide phosphate-citrate buffer was used to block endogenous peroxidase activity for 10 min at room temperature. Goat serum (5%; Fuzhou Maixin Biotech Co., Ltd., Fuzhou, China) was used to block the samples for 10 min at room temperature. The sections were then rinsed with PBS for 5 min and incubated with primary antibody against FTL (cat. no. SAB2108636; 1:500 dilution; Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) and against FTH (cat. no. SAB210862; 1:250 dilution; Sigma-Aldrich, Merck KGaA) for 12 h at 4°C. The sections were incubated with the biotinylated goat anti-rabbit secondary antibody (cat. no. SP KIT-C9; 1:250 dilution; Fuzhou Maixin Biotech Co., Ltd.) for 30 min at room temperature. The slides were stained with dianinobenzidine for 5 min at room temperature. Hematoxylin was used to counterstain the nucleus for 5 min at room temperature, followed by dehydration, and mounting.

 Evaluation of iron staining and IHC. Images of the stained sections were captured using an Olympus BX40 light microscope and CC-12 Soft-Imaging System (Olympus Corporation, Tokyo, Japan). The sections were analyzed and scored for intensity (0-3) and frequency (0-4). The intensity was scored as follows: Grade (0) negative; grade (1) weak intensity; grade (2) moderate intensity; and grade (3) strong intensity. The frequency scores were assigned when 0-25, 26-50, 51-75 and 76-100% of the tumor cells were positive, respectively. For statistical analysis, the intensity and frequency were transformed into a Composite Expression Score (CES) using the formula: CES=intensity x frequency. The range of CES was 0-12. The CES was scored as negative (0) weak positive (1-4) positive (5-8) or strong positive (9-12).

 GEO dataset. The GSE33205 (Cancer Outlier Gene Profile Sets Elucidate Pathways and patient-Specific Targets in Head
and Neck Squamous Cell Carcinoma), GSE6631 (Expression Data from Head and Neck Squamous Cell Carcinoma) and GSE27020 (Identification and Validation of a Multi-gene Predictor of Recurrence in Primary Laryngeal Cancer) datasets were obtained from the GEO (https://www.ncbi.nlm.nih.gov/). The expression values of FTH and FTL were transformed into relative expression values, and expression levels were compared between HNSCC and normal tissues, using GEO2R software (https://www.ncbi.nlm.nih.gov/geo2r).

Statistical analysis. All statistical analyses were performed using SPSS version 20.0 (IBM Corporation, Armonk, NY, USA). Quantitative data are expressed as the mean ± standard deviation. Each test was performed at least three times. A Student's t-test was used to compare the differences between two groups. One-way analysis of variance (ANOVA) was performed for the comparison of multiple groups. If the results of the ANOVA indicated significant differences, post-hoc analysis was performed with the Tukey test. The diagnostic accuracy was evaluated using receiver operating characteristic (ROC) curve analysis. A 4-fold Table $\chi^2$ test was used to determine the sensitivity and specificity of size and shape. Correlation analysis was performed using Pearson's correlation coefficient. P<0.05 was considered to indicate a statistically significant difference.

Results

SF levels are higher in patients with HNSCC and metastasis compared with patients with HNSCC without metastasis. All patients were divided into four groups: i) Inflammation;
ii) benign tumor; iii) precancerosis; and iv) cancer. The SF level in the cancer group was higher compared with the benign group (Fig. 1A). However, in male patients, no significant difference was reported among the four groups (Fig. 1B). In female patients, the SF level in the cancer group was significantly higher compared with the benign and precancerosis groups (Fig. 1C). The differences in the SF levels between male and female patients were compared, and the SF level of male patients was significantly higher compared with that of female patients (Fig. 1D). Postmenopausal women had a higher SF level compared with premenopausal women (Fig. 1E).

The differences in the SF level between male and female patients were compared in each group. In general, male patients had a higher SF level compared with female patients (Fig. 1F-I). Taking menopausal status into consideration, the SF level was reported to be higher in postmenopausal women compared with premenopausal women in the benign tumor group (t=9.377). (H) Difference in the SF level between male and female patients in the precancerosis group (t=2.716; P=0.014). (I) Differences in the SF level between male and female patients in the cancer group (t=-0.018; P=0.985). (J) Difference in the SF level between premenopausal and postmenopausal women in the cancer group (t=-2.359; P=0.03). (K) Difference in the SF level between premenopausal and postmenopausal women in the benign tumor group (t=4.147). (L) Difference in the SF level between premenopausal and postmenopausal women in the precancerosis group. (M) Difference in the SF level between premenopausal and postmenopausal women in the cancer group. *P<0.05, **P<0.01, ***P<0.001. NS, not significant; SF, serum ferritin.

Doppler ultrasonography may be less optimal for metastasis prediction compared with SF. The Doppler results of the 70 patients in the cancer group were collected to further examine the importance of SF in metastasis prediction. No statistically significant difference in SF was reported between male and female patients in the cancer group (Fig. 1I). The results of the Doppler and SF levels are presented in Table IV. No statistically significant difference in L/S ratio (Fig. 2D) and LD (Fig. 2E) was noted between these two groups, yet the SF level (Fig. 2F) exhibited a statistically significant difference. The sensitivity of LD and the L/S ratio for detecting metastasis was 60 and 20%, and the specificity was 37.5 and 10%, respectively. The ROC analysis of the SF level revealed that the area under the curve (AUC) for SF to predict cervical metastasis was 0.862, and the cutoff value of the SF level was 205.60 ng/ml (Fig. 2C).

Ferritin expression levels are higher in the tumor tissues of HNSCC with metastasis. Tissue samples of the cancer group were obtained from the pathology department to clarify whether the expression of ferritin was also upregulated in the tumor tissues of HNSCC with metastasis. IHC detection demonstrated that whether in primary tumor or lymph node
tissues, levels of FTH (Fig. 3A) and FTL (Fig. 3B) were higher in the HNSCC with metastasis group compared with the HNSCC without metastasis group. Iron staining (Fig. 3C) demonstrated that the iron content was higher in the HNSCC with metastasis group compared with the HNSCC without metastasis group, in primary tumor and lymph node tissues. Furthermore, the correlation analysis illustrated that the iron content was significantly correlated with SF, FTH and FTL (Fig. 3D).

Expression levels of FTH and FTL are upregulated in HNSCC tissues. Based on the above findings, two TMAs were purchased to investigate the differences in the protein expression levels of FTH and FTL between HNSCC and normal tissues. However, unlike the expression of ferritin in serum, the protein expression levels of FTH (Fig. 4A and B) and FTL (Fig. 4C and D) were significantly higher in HNSCC tissues compared with normal tissues.

The associated data from the GEO dataset were downloaded and analyzed. Using GSE33205 and GSE6631, the relative expression levels of FTH (Fig. 5A and B) and FTL (Fig. 5C and D) were compared between HNSCC and normal tissues. The gene expression levels of FTH and FTL were higher in HNSCC compared with normal tissues, which was consistent with the histochemical findings. Furthermore, the results of GSE27020 revealed that a high expression level of FTH was associated with poor survival in patients with HNSCC (Fig. 5E). However, no data relating to the association between FTL and survival were reported.

Discussion

Ferritin has a typical structure of a 24-subunit spherical protein encapsulating an iron oxide core (11). The subunit comprises two different types: FTH (21 kDa) and FTL (19 kDa) (12). Only FTH has the enzymatic activity to
convert Fe$^{2+}$ into Fe$^{3+}$ (13,14). The SF level was higher in male patients compared with female patients. Additionally, the SF level was higher in postmenopausal women compared with premenopausal women (15). These results are in accordance with previous findings. This may be due to menstruation, which causes a loss of ~250 mg iron per year (16). Undiminished iron may lead to a higher SF level in postmenopausal women.

Currently, the correlation between iron metabolism and tumor development has become a major concern (17,18). Numerous studies have revealed that the expression levels of ferritin are upregulated in various types of tumors, including HNSCC.
lung cancer and prostate cancer (19). The present study demonstrated that the iron content and expression levels of ferritin were higher in HNSCC compared with normal tissues. As reported by other studies, iron metabolism is vital for normal cell function (20). However, in tumor growth, endocytosis leads to the storage of a large amount of iron in cancer cells (21).

On the one hand, the expression of transferrin receptor 1 (TFR1) is upregulated in the tumor membrane and transports increased levels of iron into cancer cells (22,23). On the other hand, the major protein that transports iron out of cells is ferroportin, and its expression is always downregulated in cancer cells (24,25). Therefore, a large amount of iron is stored in cancer cells and subsequently promotes the expression of ferritin. With the development of the tumor, high levels of ferritin are secreted or leaked from damaged tumor cells, leading to a higher SF level (26). Notably, no statistically significant difference in the SF level was reported between HNSCC and benign tumors or inflammation in the present study. This may be due to the fact that in a few pathological cases, disturbance in iron metabolism may lead to a higher SF level (27). Therefore, SF may be not a potential serum marker for the early diagnosis of HNSCC.

The present study reported that the expression levels of ferritin were significantly higher in the HNSCC with metastasis group compared with the HNSCC without metastasis group. This is the first time, to the best of our knowledge, that this phenomenon has been described. A study indicated that ferritin may regulate vascular remodeling and angiogenesis. It was also demonstrated that ferritin may block the antiangiogenic effects of cleaved high-molecular-weight kininogen (HKa) through specific binding to the antiangiogenic domain of HKa (28). This may be a possible mechanism; however, whether it promotes the metastasis of HNSCC to cervical lymph nodes requires investigation.

Data from the GEO dataset demonstrated that high expression levels of FTH were associated with poor survival. A similar phenomenon has recently been described in breast cancer and astrocytic brain tumors (29,30). However, no relevant data about the prognostic value of FTL in HNSCC are available. Growing evidence suggests that artificial iron compounds may inhibit tumor growth and cell proliferation (31-33). For example, the iron oxide nanoparticle has become a research focus; this may inhibit neoplasm growth by inducing pro-inflammatory macrophage polarization (34).

A recent study has indicated that the surgical staging of cervical lymph nodes will not be replaced even by advanced imaging modalities in the next few years (35). The performance of Doppler, computed tomography, magnetic resonance imaging or positron emission tomography in the nodal staging of HNSCC is not perfect in the clinical setting. The present study revealed that SF had marked clinical importance in predicting metastasis. It is an inexpensive, routinely available
marker that may serve as a valuable tool and may improve predictive accuracy for lymph node metastasis of HNSCC.

In conclusion, ferritin may be used as a biomarker for the early diagnosis of other malignancies, yet it may not be applicable for HNSCC. However, the association of ferritin with HNSCC may help identify patients at high risk of metastasis. Ferritin may be a novel potential biomarker for detecting cervical node metastasis in HNSCC.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions
ZH, LW, CC and ZT designed the study and prepared the manuscript. LW, YH, FL, AZ and YX collected clinical samples and analyzed data. ZH, LW, FL, FW and BX conducted the experiments. AZ, YX, FW and BX critically reviewed the manuscript and supervised the study. ZH and LW wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate
The present study was approved by the ethics committee of Renmin Hospital of Wuhan University. All specimens were collected from patients who provided written informed consent.

Figure 5. Analysis of the data from the GEO dataset. The data from GSE33205 demonstrated that (A) the expression level of FTH (Gene ID: 2384396) was higher in HNSCC tissues compared with normal tissues (P=0.0397). The data from GSE6631 illustrated that (B) the expression level of FTH (Gene ID: 31697_s_at) was higher in HNSCC tissues compared with normal tissues (P=0.0010) (C) The expression level of FTL (Gene ID: 3858094) was higher in HNSCC tissues compared with normal tissues (P=0.0202). (D) The expression level of FTL (Gene ID: 35083_s_at) was higher in HNSCC tissues compared with normal tissues (P=0.0010). (E) The data from GSE27020 demonstrated that a high expression level of FTH was associated with a shorter DFS time in patients with HNSCC (Gene ID: 200748_s_at; HR=2.810 (1.403 to 6.021), P=0.0063), *P<0.05, **P<0.001. GEO, Gene Expression Omnibus; FTH, ferritin H subunit; FTL, ferritin L subunit; HNSCC, head and neck squamous cell carcinoma; DFS, disease free survival; HR, hazard ratio.
consent, in accordance with the principles of the Declaration of Helsinki and Good Clinical Practice Guidelines.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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