Ferritin is a potential tumor marker for colorectal cancer and modulates histone methylation in colorectal cancer cells

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Abstract: Purpose: Colorectal cancer (CRC) is a severe public health problem worldwide. Due to the insufficient sensitivity and/or specificity of traditional tumor markers, it is essential to identify new tumor markers to better diagnose and cure CRC. In this study, we aimed to determine whether serum ferritin could be a potential biomarker for CRC. Material and Methods: A total of 62 CRC patients were included in this study. The serum concentrations of ferritin, CEA, CA19-9 and CA125 were detected by the Cobas e601 electrochemiluminescence immunoassay system. The role of FTH1, the heavy subunit of ferritin, in CRC cells was explored using molecular biology methods. Results: We found that the serum ferritin level in CRC patients was higher than that in healthy controls, and the concentrations of serum ferritin were associated with clinicopathological features, such as the tumor stage and lymph node status of CRC patients. Interestingly, a correlation existed between ferritin and CEA, as well as CA19-9, in CRC patients, suggesting the potential of serum ferritin as a novel CRC tumor marker. Additionally, the expression of SUV39H1, a critical regulator of CRC progression and development, was modulated by the ferritin heavy subunit (FTH1), and FTH1 knockdown downregulated the expression of histone methyltransferase SUV39H1. Correspondingly, H3K9me2/3 (H3K9me2 and H3K9me3), the catalytic product of SUV39H1, was also reduced following FTH1 knockdown. Conclusions: This study revealed a novel role for ferritin in CRC diagnosis and is the first to demonstrate that SUV39H1 and H3K9me2/3 were modulated by FTH1 in CRC cells.

Keywords: Ferritin, FTH1, SUV39H1, colorectal cancer

Introduction

Colorectal cancer (CRC) is a severe public health problem worldwide. Although there have been significant improvements in early cancer screening and tumor therapy in the last several decades, the incidence and mortality rates of CRC remain high [1, 2]. Therefore, the detection of CRC at early stages is critical for reducing the incidence and mortality associated with this disease.

Endoscopic procedures, such as sigmoidoscopy and colonoscopy, are the most widely used diagnostic approaches at present [3], but the high cost, invasiveness and complexity of these procedures have impeded their further development in the surveillance of cancer onset [4, 5]. By contrast, the detection of serum tumor markers plays an increasingly important role in cancer diagnosis and prognosis because it is inexpensive, noninvasive and very convenient to perform [6]. Currently, serum markers such as carcinoembryonic antigen (CEA), carbohydrate antigen 19-9 (CA19-9) and carbohydrate antigen 125 (CA125), have been proven as valuable tools for patient monitoring [7, 8]. However, the major concern using these serum markers is the commonly insufficient sensitivity and/or specificity. It will be beneficial for us to identify new tumor markers to diagnose CRC more effectively in the early phase of tumorigenesis.

It has been demonstrated that ferritin is also correlated with CRC and might be a potential indicator for CRC diagnosis [9, 10]. Ferritin is a large protein that contains two subunit types, termed ferritin heavy chain (FTH) and ferritin light chain (FTL) subunits [11]. Some previous
studies have explored the role of ferritin in CRC, but many questions remain. Initially, the relative levels of serum ferritin in CRC patients compared with those in matched controls were inconsistent in different studies [9, 12, 13]. Additionally, the association between serum ferritin and the clinical characteristics of CRC patients was unclear. Moreover, it is necessary to study the function of ferritin in CRC cells and the molecular mechanism by which ferritin exerts its function.

In this study, we aimed to evaluate the levels of serum ferritin in CRC patients compared with those in healthy controls. The relationship between serum ferritin and the clinical characteristics, such as pathologic tumor/node/metagastasis (TNM) stage and lymph node status, of CRC patients was analyzed. The correlations between ferritin and CEA, CA19-9, CA125 were also analyzed. Finally, the role of ferritin in CRC cells, especially the function of FTH1, the heavy subunit of ferritin, in SUV39H1 regulation was explored. Overall, we wanted to clarify the clinical significance of serum ferritin in CRC diagnosis and elucidate the function of ferritin and its molecular basis in CRC cells.

Materials and methods

Patients

A total of 62 CRC patients who had undergone surgical resection at Peking University People’s Hospital from 2012 to 2018 were included in this study. All 62 included patients underwent curative surgery. Additionally, 59 healthy controls without abnormality in physical examination were included. The demographic and clinicopathological characteristics of patients are summarized (Table 1). It was a double-blind study and was approved by the Ethics Committee of our hospital.

Detection of serum ferritin, CEA, CA19-9, and CA125

The serum concentrations of ferritin, CEA, CA19-9 and CA125 were detected by the Roche electrochemiluminescence immunoassay system Cobas e601. All tests were performed according to the equipment operation procedure, and the calibration and quality control were performed sequentially to ensure the measurement accuracy. All the reagents were original kits from Roche Diagnostics. The reference intervals of these serum markers were as follows: ferritin: 30-400 ng/mL (male), 13-150 ng/mL (female); CEA: 0-4.7 ng/mL; CA125: 0-35 U/mL; CA19-9: 0-39 U/mL.

Cell culture

LoVo cells were obtained from the American Type Culture Collection (ATCC, USA). The cell line was cultured in Dulbecco’s modified Eagle’s medium (M&C GENE TECHNOLOGY, China) supplemented with 10% fetal bovine serum (FBS) (Biological Industries, Israel) and 1% antibiotics (M&C GENE TECHNOLOGY, China). They were maintained in a humidified incubator at 37°C with 5% CO₂.

RNA interference assay

The sequences of RNA interference (RNAi) oligonucleotides were as follows: negative control siRNA (NC): UUCUCGGAACGUGUCACGU; FTH1 siRNA1: GTGGCTTTGAAGAACTTTGC; FTH1 siRNA-
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Figure 1. A. CRC patients possess a higher level of serum ferritin than healthy controls. Serum ferritin in CRC patients (n = 62) and healthy controls (n = 59) was measured using the Roche Electrochemiluminescence Immunoassay system Cobas e601. The results are presented as mean values with p < 0.0001. B. ROC curve of serum ferritin as the CRC diagnostic marker. ROC, receiver operating characteristic; AUC, area under curve; 95% CI, 95% confidence interval; PPV, positive predictive value; NPV, negative predictive value.

RNA extraction

Total RNA was extracted from the LoVo cells using TRIzol reagent (Invitrogen, USA) according to the manufacturer’s instructions. The concentration and purity of RNA were evaluated by measuring the absorbance using the NanoDrop 2000 system (Thermo Scientific, USA). The ratio of the 260/280-nm range was between 1.8 and 2.0.

Protein extraction

Equal numbers of harvested cells were washed with PBS and were centrifuged at 12,000 rpm at 4°C for 30 seconds. Next, the cell pellet was resuspended in 30 μl (per 10⁶ cells) of 2× protease inhibitor buffer consisting of 1 cocktail protease inhibitor pellet (Roche Holding AG, Switzerland) in 3.5 ml of PBS, and an equal volume of 2× SDS loading buffer (950 μl of Laemmli buffer + 50 μl of 2-mercaptoethanol) was added to the resuspended cells. The samples were boiled at 100°C for 10 min and then pelleted by centrifuging at 12,000 rpm at 4°C for 15 min.

SDS-PAGE and western blotting

Western blotting was used to evaluate the relative protein levels as previously described [14]. Equal amounts of proteins were size fractionated on SDS-PAGE gels and then were incubated with primary antibodies and secondary antibodies sequentially. The primary antibodies used were anti-FTH1 (Abcam, UK), anti-β-actin (ZSGB-BIO, China), anti-KDM2A (ABclonal Technology, USA), anti-KDM3A (Abcam, UK), anti-SUV39H1 (Cell Signaling Technology, USA), anti-G9a (Sigma-Aldrich, USA), anti-H3K9me (ABclonal Technology, USA), anti-H3K9me2 (ABclonal Technology, USA), anti-H3K9me3 (ABclonal Technology, USA), and anti-H3 (Abcam, UK).

Statistical analysis

All analyses were carried out using GraphPad Prime 5.01 software. All the data were expressed as the mean values unless otherwise stated. The results between different groups were compared using Student’s t-test for values from a Gaussian distribution and the Mann-Whitney test for values that did not follow a Gaussian distribution. The Pearson correlation coefficients (r) were estimated between the serum ferritin and traditional tumor marker levels. All the statistical tests were 2-tailed. A p value < 0.05 was considered to be statistically significant.
Table 2. Comparison of serum ferritin in patients with different clinical characteristics

<table>
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<tr>
<th>Characteristics</th>
<th>Serum ferritin (ng/ml)</th>
<th>p</th>
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<tbody>
<tr>
<td>Age, y</td>
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<td>0.9941</td>
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<tr>
<td>&lt; 65</td>
<td>290.9±380.1</td>
<td></td>
</tr>
<tr>
<td>≥ 65</td>
<td>241.5±324.5</td>
<td></td>
</tr>
<tr>
<td>Sex</td>
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<tr>
<td>Male</td>
<td>320.8±393.3</td>
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<tr>
<td>Female</td>
<td>208.2±291.4</td>
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<tr>
<td>Tumor Size</td>
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<tr>
<td>&lt; 5 cm</td>
<td>149.6±162.2</td>
<td></td>
</tr>
<tr>
<td>≥ 5 cm</td>
<td>361.6±429.4</td>
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<tr>
<td>TNM stage</td>
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<tr>
<td>I-II</td>
<td>149.4±185.6</td>
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</tr>
<tr>
<td>III-IV</td>
<td>410.9±444.3</td>
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</tr>
<tr>
<td>Differentiation</td>
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<tr>
<td>Well</td>
<td>203.8±204.2</td>
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</tr>
<tr>
<td>Moderate</td>
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<tr>
<td>Poorly</td>
<td>497.2±544.9</td>
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<tr>
<td>Venous permeation</td>
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<tr>
<td>Negative</td>
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<tr>
<td>Positive</td>
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<td>Lymph node status</td>
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</table>

*p < 0.05.

Results

The serum level of ferritin in colorectal cancer patients was higher than that in healthy controls

Sixty-two CRC patients and 59 healthy controls were enrolled in this study. The serum ferritin level of each individual was measured using the Cobas e601 system. We found that CRC patients possessed a higher level of serum ferritin than healthy controls, and the difference was highly significant (p < 0.0001) (Figure 1A). The average levels of serum ferritin in CRC patients and healthy controls were 259.03 and 77.57 ng/ml, respectively. Next, to clarify the diagnostic value of serum ferritin, ROC (receiver operating characteristic) analysis was performed, and the AUC (area under curve) was calculated. As shown in Figure 1B, the AUC of serum ferritin was 0.7166, indicating its diagnostic value for CRC patients. The best cutoff value was 48.45 ng/ml, and the sensitivity and specificity at the cutoff point was 72.58% and 61.02%, respectively.

The serum ferritin levels were related to the TNM stage and lymph node status of CRC patients

Because the level of serum ferritin was upregulated in CRC patients, it was expected that a relationship between the serum ferritin levels and clinical characteristics of CRC patients exists. We found that the level of serum ferritin in TNM stage III-IV patients was significantly higher than that in stage I-II CRC patients (p < 0.05) (Table 2). Additionally, the serum ferritin levels in the groups positive (N1+N2) and negative (N0) for lymph node metastasis were also different, and the level of serum ferritin in the N1+N2 group was higher than that in the N0 group, with statistical significance (p < 0.05) (Table 2). However, no statistically significant relationship was found between the serum ferritin level and tumor differentiation, venous permeation, distant metastasis nor tumor size. Collectively, these findings suggest that there are tight correlations between the serum ferritin level and some characteristics, such as TNM stage and lymph node status of CRC patients.

The ferritin levels positively correlated with CEA and CA19-9 in the serum of CRC patients

Because the level of serum ferritin, as well as that of traditional CRC markers, including CEA, CA125 and CA19-9, was increased in CRC patients, we hypothesized that serum ferritin may be correlated with CEA, CA125 or CA19-9. The serum levels of CEA, CA125 and CA19-9 were determined by the Cobas e601 system, and correlation analysis was conducted. Interestingly, we found that serum ferritin was positively correlated with CEA and CA19-9 (r > 0)
and both correlations were significant ($P < 0.05$) (Figure 2A, 2C). For CA125, however, the correlation was not significant (Figure 2B). These data suggest that serum ferritin was related to traditional tumor markers in CRC patients, implying that serum ferritin might also be a CRC tumor marker.

**FTH1, the heavy subunit of ferritin, promoted the expression of histone methyltransferase SUV39H1**

Next, we investigated the role of ferritin in CRC cells. Because the main function of ferritin is the storage of iron [11], and iron is tightly associated with histone methylation [15], we hypothesized that ferritin regulates histone methylation. To verify the hypothesis, FTH1, the heavy subunit of ferritin, was first knocked-down in CRC LoVo cells by RNA interference, and the FTH1-knockdown induced cell model was successfully established (Figure 3A, 3B). Next, the relative protein levels of some histone methyltransferases and histone demethylases following FTH1 knockdown were detected by Western blotting. As shown in Figure 3C, the protein level of histone methyltransferase SUV39H1 was downregulated by FTH1 knockdown. However, the levels of other histone methylation enzymes, such as histone methyltransferase G9a, histone demethylase KDM2A and KDM3A, did not change significantly. To elucidate whether FTH1 regulates the mRNA expression of SUV39H1, the relative mRNA level of SUV39H1 in response to FTH1 knockdown was detected by real-time PCR. As shown in Figure 3D, the mRNA level of SUV39H1 was also downregulated significantly by FTH1 knockdown, indicating that FTH1 regulates SUV39H1 expression at the transcriptional level.

Because SUV39H1 is a histone methyltransferase mainly catalyzing the formation of H3-K9me2 (dimethylation at H3K9) and H3K9me3 (trimethylation at H3K9) [16], we explored whether the levels of H3K9 methylation were regulated by FTH1 knockdown. As shown in Figure 4, reduced H3K9me2/3 (H3K9me2 and

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**Figure 2.** Significant correlation between serum ferritin and CEA (A), as well as CA19-9 (C), but not CA125 (B) in CRC patients. Scatter plots with a linear fit are shown, and Pearson $r$ and $p$ values are listed. $P$ values $< 0.05$ are regarded as significant.
H3K9me3) was observed in response to FTH1 knockdown, while the level of H3K9me did not change significantly. Together, these results suggest that ferritin modulates SUV39H1 expression at the transcription level and H3K9 methylation in CRC cells.

**Discussion**

In this study, ferritin was identified as a potential tumor marker for CRC. Our data indicate that the serum ferritin level in CRC patients was higher than that in healthy controls, and the
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Figure 4. Decreased H3K9me2/3 expression in response to FTH1 knockdown. A. LoVo cells were transfected with NC, FTH1 siRNA#1 or FTH1 siRNA#2. At 48 hours after transfection, the relative levels of H3K9me, H3K9me2 and H3K9me3 were detected by Western blotting. B. The bands from immunoblotting were scanned and quantified, as shown in histograms (means ± s.d., n = 3).

level of serum ferritin was likely associated with cancer development and progression because of its correlation with the TNM stage and lymph node status of CRC patients. Interestingly, a linear positive correlation was found between serum ferritin and CEA, as well as CA19-9, implying the diagnostic potential of ferritin as a tumor marker. Additionally, FTH1 knockdown induced a decrease in SUV39H1 expression and its catalytic product H3K9me2/3 correspondingly, suggesting a tight relationship between iron metabolism and histone methylation.

SUV39H1 has been extensively reported to play an important role in CRC. It is a critical epigenetic factor with the regulatory function of gene transcription [22]. It was demonstrated that SUV39H1, as well as its product H3K9me3, was overexpressed in CRC tissues and activated the cell invasion and migration of CRC cells [23, 24]. Additionally, Fas, a critical factor in cancer immune surveillance, was transcriptionally silenced by SUV39H1-induced H3K9me3 in metastatic human colon carcinoma, resulting in immune escape and 5-fluorouracil chemoresistance. In contrast, inhibition of H3K9me3 induction in the Fas promoter region restored Fas expression and then overcame immune evasion and metastatic colon carcinoma resistance to 5-fluorouracil [25]. However, the regulation of SUV39H1 expression itself in CRC is largely unknown. Our study revealed that SUV39H1 expression and H3K9me2/3 levels were

Ferritin has been reported to be associated with different types of cancer. Initially, it was demonstrated that the level of serum ferritin in lung cancer patients was significantly higher than that in controls, and there was a statistically significant correlation between the serum ferritin level and gender, smoking, regional lymph node metastasis or distant metastasis [17, 18]. Additionally, it was demonstrated that elevated iron storage by increased ferritin may contribute to the carcinogenesis of breast cancer because iron overload favors the production of reactive oxygen species, lipid peroxidation, and DNA damage [19, 20]. Our study contributes evidence that the serum ferritin level was elevated in CRC patients, and it was correlated with the clinicopathological characteristics and traditional tumor markers of CRC. Further study indicated that the expression of SUV39H1, a critical regulator of CRC development and progression, was modulated by the heavy subunit FTH1 of ferritin.

The relative level of serum ferritin in colorectal cancer patients varies in different studies. Some studies have shown no significant differences in the serum ferritin levels in patients with CRC or adenomas vs. those with negative colonoscopy [12, 21]. However, another study reported that the serum ferritin levels in patients with colorectal cancer were significantly decreased compared with those in controls [13]. Additionally, some CRC patients were reported to have raised ferritin levels, and patients with high ferritin levels had a shorter survival than those with normal levels [9]. Consistently, serum ferritin was also elevated in CRC patients compared with those in healthy controls in our study. The difference in the relative ferritin level among the different studies might be due to the geographical regions and races of the included patients. The included patients in our study were from the Han population in the northern part of China, which is different from other studies.
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reduced by FTH1 knockdown, suggesting the role of ferritin/FTH1 in the regulation of SUV39H1 expression and CRC progression.

In summary, our data described the clinical significance of serum ferritin and function of FTH1 in the regulation of SUV39H1 expression. This study indicates that serum ferritin might be a potential tumor marker for the diagnosis of CRC and provides a useful strategy with which to design anticancer therapies and, eventually, cure cancer by targeting the FTH1-SUV39H1 pathway.

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Disclosure of conflict of interest

None.

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