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Decreased expression of ferritin light chain in osteosarcoma and its correlation with epithelial-mesenchymal transition

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Abstract. – OBJECTIVE: The purpose of this study was to detect ferritin light chain (FTL) expression level in osteosarcoma (OS), and to clarify whether FTL could offer additional help in diagnosis or therapy.

MATERIALS AND METHODS: First, we assessed FTL level in OS tissues and cells through GEO dataset and tissue microarrays (TMAs). Then, we overexpressed FTL expression in MG-63 cell line. Lastly, we detected the expression of EMT-related signal pathway proteins to study its underlying molecular mechanisms.

RESULTS: GEO dataset and TMAs showed that FTL was down-regulated in OS. After FTL was overexpressed, the proliferation, migration and invasion abilities of OS cells were significantly reduced. Moreover, after FTL overexpressing, the levels of CDH2 and Vimentin were down-regulated with CDH1 up-regulated.

CONCLUSIONS: We revealed that FTL (1) is lower in OS then in normal tissue, (2) is related to metastasis, survival period, and therapeutic response, and (3) may be a tumor-inhibiting factor owing to its inhibition of EMT in OS.

Key Words:

Ferritin light chain, Osteosarcoma, Tumor marker, EMT, GEO dataset.

Introduction

Osteosarcoma (OS), which always occurred in the long tubular bones, is a main malignant tumor happened in the skeleton[1]. It is the most common primary malignant bone tumor[2]. In children and adolescents, OS is an aggressive malignant mesenchymal tumor with a relatively high rate of morbidity and mortality[3,4]. The main cause is that OS cells proliferate rapidly and invade nearby tissues[5]. In the past few years, great progress has been made, but the molecular and pathological mechanisms of OS is still not been fully elucidated[6]. Hence, more investigates about OS are necessary and meaningful.

Ferritin has been studied for nearly 80 years and remains an interesting molecule[7]. As the most important iron storage protein, ferritin has been utilized as a marker of iron stores with a long history[8]. It has two functionally distinct subtypes of FTL and ferritin heavy chain (FTH)[9]. Nowadays, a growing number of papers suggests that both of the subunits are closely related to the growth of malignant tumor cells[10]. Despite these findings, the role of FTL in OS remains largely unknown.

In this study, we first assessed FTL level in OS samples through GEO dataset and TMAs. Then, we did cell experiments to study its underlying molecular mechanisms. Our findings suggest that FTL is down-regulated in OS and is a potential regulator of the EMT process. Therefore, we revealed the important role played by FTL in OS.

Materials and Methods

Cell Culture and Main Reagents

The human osteosarcoma cell line, MG-63 was cultured in Roswell Park Memorial Institute-1640 (RMPI-1640; Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS) at 37°C, 5% CO_2. We obtained FBS from HyClone (Logan, UT, USA). We purchased RPMI-1640 media and 0.25% trypsin solution from Invitrogen. For Western blotting, we used the cells in the logarithmic growth phase.

Gene Expression Profiles

GEO dataset GSE39262 (human sarcoma cell lines and untransformed cells), GSE21257 (Genome-wide gene expression profiling on pre-chemotherapy biopsies of osteosarcoma patients who

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developed metastases within 5 years and patients who did not develop metastases within 5 years) and GSE19276 (osteosarcoma is characterized by impaired osteoclastogenesis and antigen presentation compared to normal bone) were employed to investigate the expression difference of FTL between osteosarcoma and normal tissue, and also, to explore the correlation between FTL level and metastasis, therapeutic response, and survival time.

Patients Selected and TMA

Two independent TMAs (Alena Biotechnology co., LTD, Xi'an, China) (Production number: BO244d and OS804b) were purchased. The TMAs consisted of 11 bone tissues and 41 OS tissues (male: n=38 men; female: n=14). The mean age was 37.5 years old (ranged from 11 to 84 years old) (Table I).

Immunohistochemical Staining

We deparaffined TMAs thrice using standard pure xylene for 15 min at room temperature. Then, TMAs were hydrated in graded alcohols, followed by washing with phosphate-buffered saline (PBS). We performed antigen retrieval in boiling citrate buffer (pH 6.0) for 15 min. TMAs were cooled at room temperature in the buffers. After washing with PBS for 5 min thrice, we used 0.3% hydrogen peroxide phosphate-citrate buffer to block endogenous peroxidase activity for 10 min. TMAs were rinsed with PBS for 5 min and incubated with primary antibody FTL (dilution 1:500; Sigma-Aldrich, St. Louis, MO, USA) for 12 h at 4°C. We incubated TMAs with Poly-HRP Goat anti-rabbit (Maixin Bio, Fuzhou, China) for 30 min and stained slides with diaminobenzidine for 5 min. To counterstain the nucleus, hematoxylin was used, followed by dehydration and mounting.

Evaluation of Immunohistochemical Staining

FTL showed both cytoplasmic and membrane expression. Images of stained TMAs were taken using an Olympus BX40 microscope and CC-12 Soft-Imaging System (Olympus, Tokyo, Japan). We analyzed TMAs and scored them for intensity (0-3) and frequency (0-4). Of note, the intensity was scored as follows: grade (0), negative; grade (1), weak intensity; grade (2), moderate intensity; and grade (3), strong intensity. Furthermore, the frequency scores of 0-4 were respectively assigned when 0%-25%, 26%-50%, 51%-75%, and 76%-100% of tumor cells were positively stained. To transform the intensity and frequency into a composite expression score (CES), we used the following formula: CES = Intensity ´ Frequency for statistical analysis. The range of CES was from 0 to 12. The CES was scored as negative (0), weak positive (1-4), positive (5-8), strong positive (9-12). All the staining scores were analyzed and determined by two experienced pathologists.

Gene Transfection

FTL cDNA was cloned into the plasmid. After amplification and DNA sequence confirmation, this plasmid was used to overexpress FTL in MG-63 cells. Briefly, cells were grown and stably transfected with plasmid-FTL or plasmid-empty for control. Cells were selected 72 h after transfection with 1.5 ng/mL puromycin and named MG-63/CTRL, MG-63/FTL.

Western Blotting

The total cell lysate was performed according to standard instructions. The lysates were resolved using 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene difluoride (PVDF) membranes and immunoblotted with primary antibodies against FTL, N-Cadherin (CDH2), Vimentin, E-Cadherin (CDH1), and GAPDH. Following incubation with secondary antibodies, the protein bands were detected using an enhanced chemiluminescence (ECL) reagent (Thermo Fisher Scientific, Rockford, IL, USA).

Table I. Details of the characteristics of Tissue microarrays (TMAs).

Variable N = 52	Gender		Age (years)	
	Male	Female	Range	Mean
Bone N = 11	10	1	41~84	61.6
Osteosarcoma N=41	28	13	11~64	31.0

Cell Proliferation

MG-63 cells were seeded at 1×103/well in 96-well plates in RPMI 1640 medium supplemented with 10% FBS. The cells were transfected after 24 h, and we determined the proliferation at 0, 24, 48, and 72 h post-transfection using the Cell Counting Kit-8 (CCK-8), according to the manufacturer's instructions.

Transwell Assay

Cell migration and invasion assays were performed using transwell with Matrigel-coated inserts (BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's protocol. In brief, 1×10^5 cells were suspended in 200 mL serum-free RPMI-1640 medium. In the upper chamber, the cells were seeded in Matrigel-coated inserts; the lower chamber contained the RPMI 1640 medium with 10% FBS as a chemoattractant. After 48-h incubation at 37°C in 5% CO_2 humidified atmosphere, cells not penetrating the membrane were removed using cotton swabs. Conversely, cells successfully migrating to the membranes' bottom surface were fixed with 4% polyoxymethylene and stained with 0.1% crystal violet for 20 min, followed by counting under a microscope at a magnification of ´100.

Statistical Analysis

All statistical analyses were performed using the statistical package for social sciences (SPSS) version 20.0 software (IBM, Armonk, NY, USA). The data was downloaded from GEO dataset, and the differences were analyzed. FTL expression was compared with the *t*-test. Probability values < 0.05 were considered statistically significant.

Results

Analysis of the Data from GEO Dataset

Through GEO dataset we analyzed the expression level of FTL in OS, and also analyzed the correlation between FTL and metastasis, therapeutic response, survival time of OS. GSE39262 showed that FTL was more down-regulated in tumor than in normal cells (Figure 1A). GSE21257 showed that low FTL level was related to both of tumor metastasis (Figure 1B) and short survival period (Figure 1D). GSE19276 showed that higher FTL level was correlated with better therapeutic response (Figure 1C).

Analysis of FTL Expression in TMAs

To investigate the expression level of FTL in clinic samples of OS, we used two TMAs to have a subset analysis. BO244d consisted of 11 bone tissues and 1 OS tissue, and OS804b 40 OS tissues. The CES scores of every sample of the TMAs were measured. We chose the images of FTL expression from the TMAs, representative images were shown in Figure 2A. FTL expression in OS was significantly lower than normal bone tissue (Figure 2B). The result was also consistent with what we analyzed from the GEO dataset.

FTL Overexpressing in MG-63

MG-63 cells were stably transfected with plasmid-FTL or plasmid-empty (Figure 3A). After FTL was overexpressed, both of protein (Figure 3B) and mRNA (Figure 3C) were up-regulated in MG-63/FTL cells. Moreover, CCK-8 revealed that the proliferation of MG-63/FTL cells inhibited in a time-dependent manner compared with the control cells (Figure 3D).

Analysis of the Transwell Assay Results

To further study the effect of FTL in MG-63 cells, cell migration and invasion abilities were evaluated. The results showed that in MG-63/FTL cells the migration (Figure 4A) and invasion (Figure 4B) abilities were significantly reduced than in MG-63 or MG-63/CTRL cells.

The Expression of EMT-Related Signal Pathway Proteins

Through Western blotting, we detected the expression of EMT-related signal pathway proteins, including CDH2, Vimentin, and CDH1. The results revealed that after FTL overexpressing, the levels of CDH2 and Vimentin were down-regulated with CDH1 up-regulated (Figure 5A). Then, we analyzed the correlation of these proteins through GSE21257; we found that FTL was closely related to the expression of these three genes (Figure 5B-5C).

Discussion

OS always occurred directly from the immature bone or osteoid tissue forming by tumor cells. Most patients diagnosed with OS are less than 20 years old[11]. At present, the primary pharmacodynamic strategy to prevent OS progression is neoadjuvant chemotherapy[12]. Although treatments have progressed over the past years, the

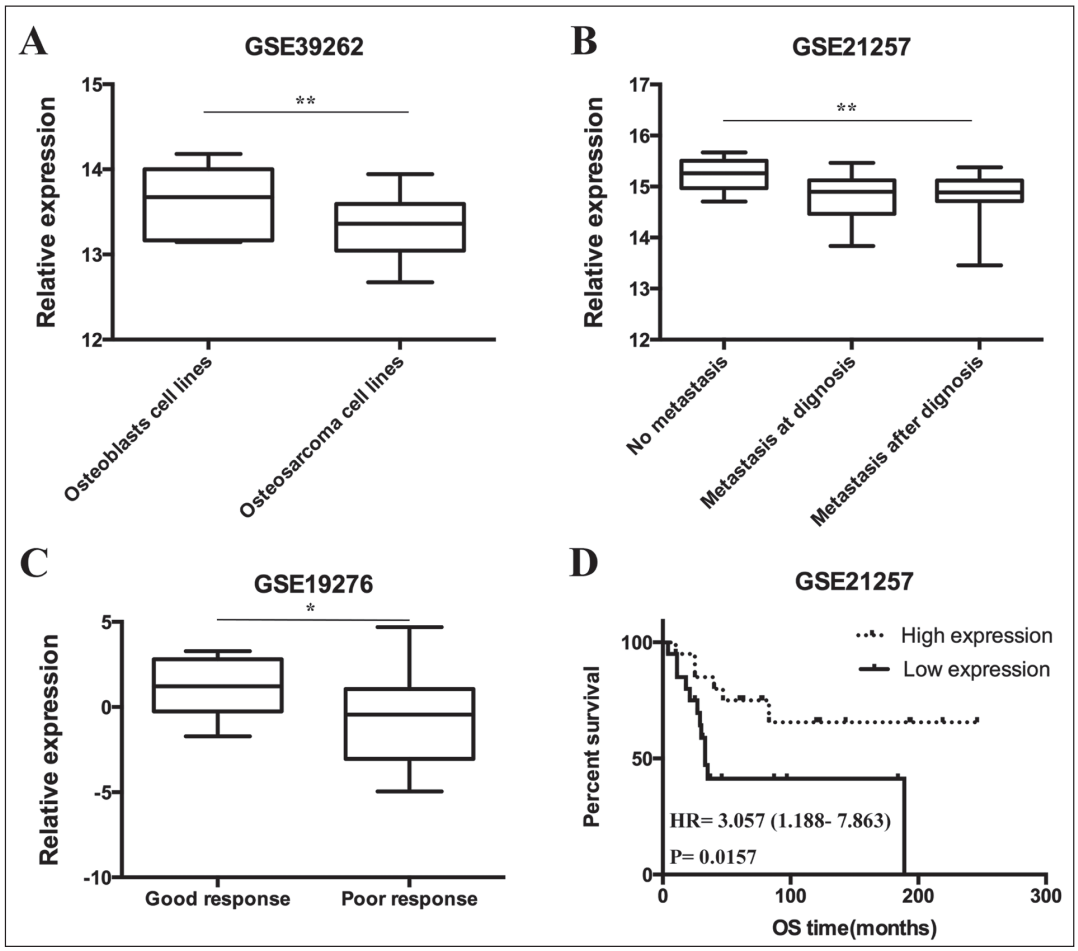

Figure 1. Analysis of FTL expression in GEO dataset. FTL was more down-regulated in tumor than in normal cells (***A***, $p < 0.05$). Low FTL level was related to both of tumor metastasis (***B***, $p < 0.05$) and short survival period (***D***, $p < 0.05$). Higher FTL level was correlated with better therapeutic response (***C***, $p < 0.05$).

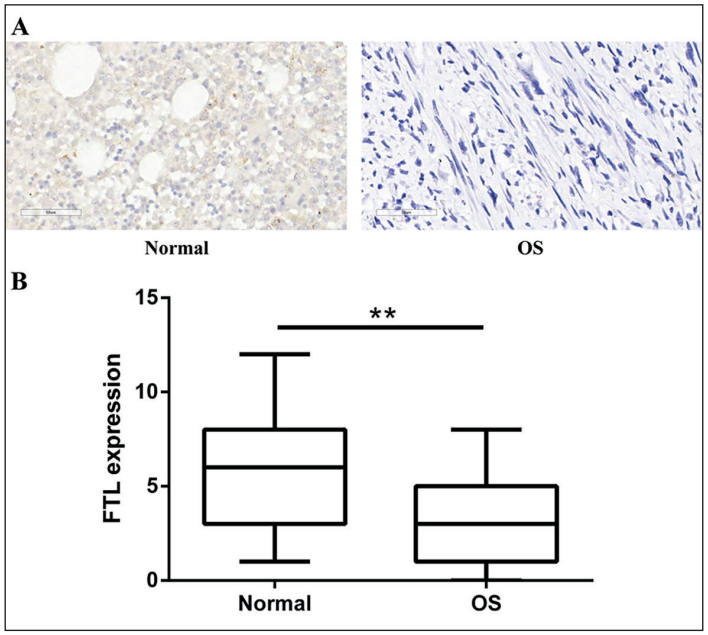

Figure 2. Analysis of FTL expression in TMAs. Representative images of FTL expression from the TMAs were shown in (***A***, × 200 magnification). FTL expression in OS was significantly lower than normal bone tissue (***B***, $p < 0.05$).

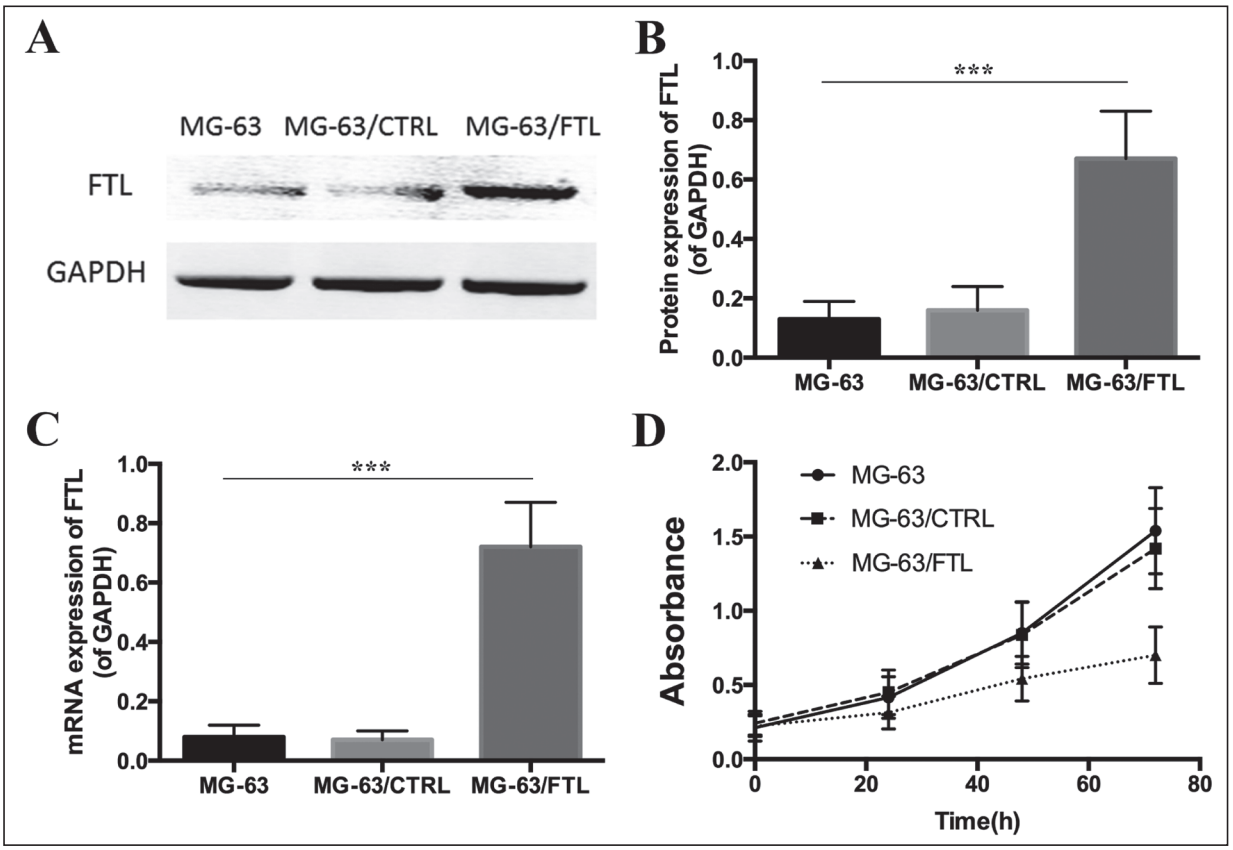

Figure 3. FTL expression and cell proliferation in FTL overexpressing cell line. MG-63 cells were stably transfected with plasmid-FTL or plasmid-empty (***A,***). After FTL was overexpressed, both of protein (***B,*** $p < 0.05$) and mRNA (***C,*** $p < 0.05$) of FTL were up-regulated in MG-63/FTL cells. CCK-8 revealed that the proliferation of MG-63/FTL cells inhibited in a time-dependent manner (***D,*** $p < 0.05$).

5-year survival rate of OS is approximately only 70 percent[13]. This proportion is not satisfactory. Most poor prognoses are due to the metastases which usually occur in the lung[14]. The 5-year survival rate of OS patients with pulmonary metastasis is just about 28 percent[15]. Because of this, we

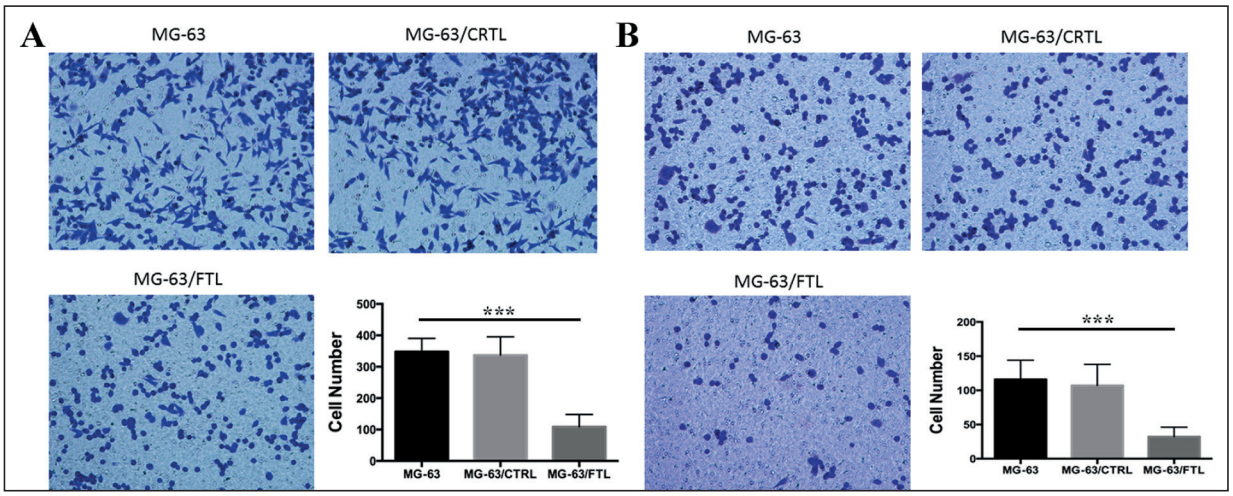

Figure 4. Analyze the results of transwell assay. In MG-63/FTL cells the migration (***A,*** $p < 0.05$) and invasion (***B,*** $p < 0.05$) abilities were significantly reduced than in MG-63 or MG-63/CTRL cells.

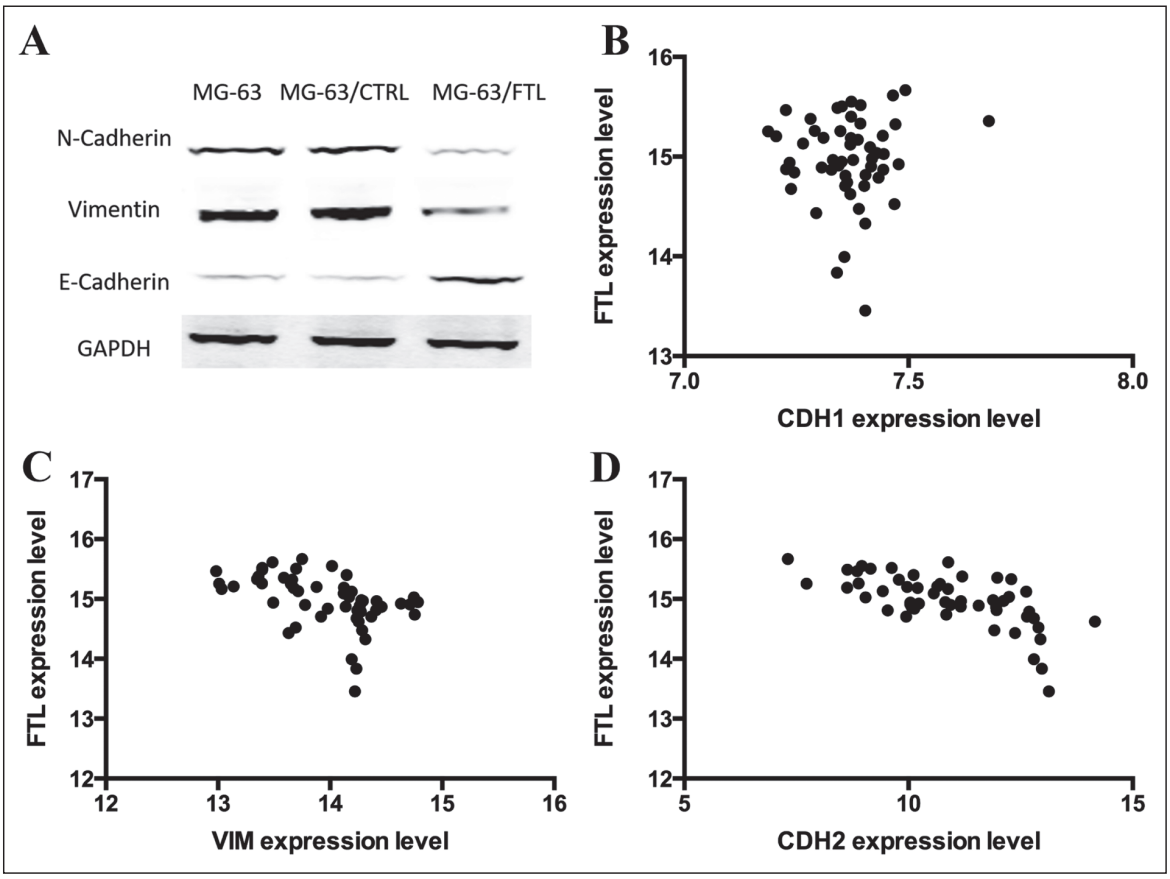

Figure 5. The expression of EMT-related signal pathway proteins. After FTL overexpressing, the levels of CDH2 and Vimentin were down-regulated with CDH1 up-regulated (***A,*** $p < 0.05$). Data from GSE21257 revealed that FTL expression was closely related to the expression of CDH1 (***B,*** $p < 0.05$), Vimentin (***C,*** $p < 0.05$) and CDH2 (***D,*** $p < 0.05$).

designed this study to investigate the role of FTL in OS. Our goal was to clarify whether FTL could offer additional help in the diagnosis or therapy.

It has been reported for a long time that the primary iron storage protein is ferritin. Tumor ferritins are composed of its two subunits[16]. Growing evidence suggests that ferritin expression level is dysregulated in many kinds of tumors, such as glioblastoma and alimentary tract carcinoma[17,18]. However, there is no research on the role of ferritin, let alone FTL, in OS. FTL lacks enzymatic activity and can't oxidize ferrous iron into ferric iron. Its most important role is to line the ferritin cavity[19].

In the present study, we analyzed FTL expression in OS through GEO dataset and TMAs. The results showed that FTL was more down-regulated in tumor than in normal tissue and that FTL was related to metastasis, survival period, and therapeutic response. The down-regulation of FTL was also found in the first stage of lung cancer and hepatocellular carcinoma[20,21]. However, in glioblastoma multiforme and human metastatic melanoma cells, FTL was over-expressed[17,22]. This different expression status may be related to tumor heterogeneity.

Furthermore, Wu et al[17] revealed that FTL could promote the proliferation of tumor cell *via* GADD45/JNK pathway. We observed the contrary results. After FTL gene transfection, OS cell proliferation, migration, and invasion were significantly attenuated. From it, we suspect that FTL could promote or inhibit tumor growth through different mechanisms.

Then, we conducted protein detection through Western blotting to explore the potential mechanism. The results revealed that CDH2 and Vimentin were down-regulated with CDH1 up-regulated in MG-63/FTL cells. The data from GSE21257 further verified the correlation of these genes which play essential roles in EMT-related signal pathways.

EMT refers to the process that cells transit from epithelial to mesenchymal phenotype[23]. Emerging data suggest that EMT progression is critical in the occurrence and development of many tumors[24,25]. The marker proteins of EMT include E-cadherin, Zonula occludens-l, Vimentin, and N-cadherin. In EMT process the first two proteins are down-regulated with the last two up-regulated[26]. In this study, we showed that after FTL overexpressing, the levels of CDH2 and Vimentin were down-regulated with CDH1 up-regulated. Overexpression of FTL was shown to inhibit EMT progression in OS cells.

Conclusions

We showed that FTL 1) is lower in OS then in normal tissue, 2) is related to metastasis, survival period and therapeutic response, and 3) may be a tumor-inhibiting factor owing to its inhibition of EMT.

Ethics Approval

It was not required because what we used were two independent TMAs (Alena Biotechnology co., LTD, Xi'an, China).

Conflict of Interest

The Authors declare that they have no conflict of interests.

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