

Downregulation of FTL decreases proliferation of malignant mesothelioma cells by inducing G₁ cell cycle arrest

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Abstract. Pleural malignant mesothelioma is a malignant tumor with a poor prognosis that is strongly associated with asbestos exposure during its development. Because there is no adequate treatment for malignant mesothelioma, investigation of its molecular mechanism is important. The ferritin light chain (FTL) is a subunit of ferritin, and its high expression in malignant tumors, including malignant mesothelioma, has recently been reported; however, its role in malignant mesothelioma is unclear. The purpose of the present study was to clarify the function of FTL in malignant mesothelioma. The expression levels of FTL in malignant mesothelioma were examined using the Cancer Cell Line Encyclopedia database and our previous data. The short interfering (si)RNA against FTL was transfected into two mesothelioma cell lines, ACC-MESO-1 and CRL-5915, and functional analysis was performed. Expression of p21, p27, cyclin-dependent kinase 2 (CDK2) and phosphorylated retinoblastoma protein (pRb) associated with the cell cycle were examined as candidate genes associated with FTL. The expression levels of the FTL mRNA were higher in malignant mesothelioma compared with other tumors in the Cancer Cell Line Encyclopedia database, and among other genes in our previous study. Reverse transcription-quantitative PCR and western blotting demonstrated suppression of FTL expression in two cell lines transfected with FTL siRNA compared with cells transfected with negative control (NC) siRNA. In the two cell lines transfected with FTL siRNA, proliferation was significantly suppressed, and cell cycle arrest was observed in the G₁ phase. The levels of p21 and p27 were increased, while those of CDK2 and pRb were decreased compared with NC. However, no significant

differences in invasion and migration ability were revealed between FTL siRNA-transfected cells and NC. In conclusion, FTL may increase the proliferative capacity of malignant mesothelioma cells by affecting p21, p27, CDK2 and pRb, and promoting the cell cycle at the G₁ phase.

Introduction

Malignant mesothelioma is an extremely aggressive tumor with a poor prognosis; its occurrence is increasing worldwide, primarily due to past and/or present occupational and/or environmental asbestos exposure (1). Malignant mesothelioma is still predominant in developed countries, including Japan, but a shift in disease occurrence is anticipated, as asbestos use has recently increased in developing countries (2). The molecular mechanism of carcinogenesis and progression of malignant mesothelioma remains unclear, and effective therapy has not yet been established. Ferritin is a multifunctional protein that functions intracellularly or extracellularly and contributes to proliferation, angiogenesis, immune suppression, and iron delivery in both non-tumor and tumor cells. Ferritin consists of two subunits, light chain and heavy chain, and these subunits are functionally and genetically distinct (3). However, it has been reported that ferritin light chain (FTL) and ferritin heavy chain (FTH) may be involved in mesothelioma in relation to iron metabolism (4,5), particularly FTH (6). High protein or mRNA expression of FTL is associated with tumor malignancy in colorectal cancers; an increase in FTL is negatively correlated with survival and promotes migration, invasion, and metastasis (7). In gastric cancers, patients with low FTL expression have longer overall survival and recurrence-free survival (8). In glioblastomas, FTL expression is higher in glioblastoma patients than in low-grade glioma patients, and knockdown of FTL reduces cell growth (9). Increase FTH expression is reported to be associated with malignant tumor grade of renal cell carcinoma (10), FTL and FTH have no relation to each other in previous reports of tumorigenesis of malignant tumors. Mohr *et al* reported that the expression levels of FTL mRNA were increased among 302 overexpressed genes in malignant mesothelioma (11); however, the molecular role of FTL in malignant mesothelioma remains unclear. In this study, we investigated the role of FTL in malignant mesothelioma.

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Materials and methods

Investigation of expression levels of FTL in malignant mesothelioma. The expression levels of FTL in malignant mesothelioma were examined using the Cancer Cell Line Encyclopedia (CCLE, Broad Institute, <https://www.broadinstitute.org/ccle/>) (12), which shows gene expression levels in various tumors, on October 30, 2020. We also used the DepMap Portal (Broad Institute, <https://depmap.org/portal/>) to search for FTL expression levels in mesothelioma cell lines, distinguishing between subtypes. We reanalyzed the gene expression data that we had previously obtained for six epithelioid mesotheliomas and six lung adenocarcinomas (13).

Mesothelioma cell lines. The ACC-MESO-1 cell line was purchased from RIKEN BioResearch Center (Tsukuba, Japan), and the CRL-5915 cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). Mesothelioma cells were maintained in Roswell Park Memorial Institute 1640 medium with GlutaMAX and sodium pyruvate (RPMI-1640) supplemented with 1% kanamycin/fungizone and 5% fetal bovine serum (FBS) in a humidified incubator with 5% CO₂ at 37°C (all purchased from Gibco/Thermo Fisher Scientific K.K., Tokyo, Japan).

Transfection of mRNA inhibitors to mesothelioma cells. Mesothelioma cell lines were transfected with Silencer Select siRNAs to reduce *FTL* mRNA levels (sense: CCUGGAGAC UCACUCCUATT; antisense: UAGGAAGUGAGUCUC CAGGAA; Thermo Fisher Scientific, Tokyo, Japan) and negative control (NC; Select Negative Control No. 1 siRNA, Cat# 4390843, Thermo Fisher Scientific K.K.) using Lipofectamine RNAiMAX and Opti-MEM (Thermo Fisher Scientific K.K.) according to the manufacturer's protocols.

Real-time reverse transcription polymerase chain reaction. The two mesothelioma cell lines (2x10⁵ cells) were transfected with 25 pmol of *FTL* siRNA or NC siRNA in 6-well plates for 72 h. RNA was extracted from the cells using Maxwell RSC SimplyRNA Cells Kits on a Maxwell RSC Instrument (Promega Japan) according to the manufacturer's protocols. The extracted RNA was reverse transcribed with SuperScript IV VILO Master Mix (Thermo Fisher Scientific K.K.) and amplified using PowerUp SYBR Green Master Mix (Thermo Fisher Scientific K.K.) on an AriaMx Real-Time PCR System (Agilent Technologies, Tokyo, Japan). Relative expression levels were calculated using the comparative Cq method. Expression levels were normalized to that of glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). The primer sequences were as follows: forward, 5'-GGCTTCTATTTGACCGC GA-3'; *FTL* reverse, 5'-TTTCATGGCGTCTGGGGTTT-3'; *GAPDH* forward, 5'-ACAACCTTGGTATCGTGGAAG-3'; *GAPDH* reverse, 5'-GCCATCACGCCACAGTTTC-3'.

Western blot analysis. A total of 1.5x10⁵ cells of the two mesothelioma cell lines were transfected with 25 pmol *FTL* siRNA or NC siRNA in 6-well plates for 48 h. Cell lysates were obtained using the RIPA Lysis Buffer System (Santa Cruz Biotechnology), and the protein concentration in the lysates was measured using a Qubit Fluorometer (Thermo

Fisher Scientific K.K.). Thirty micrograms of protein were electrophoresed on a sodium dodecyl sulfate-polyacrylamide gel (SureCast Acrylamide Gel, Thermo Fisher Scientific K.K.) at 200 V for 40 min and immediately transferred onto polyvinylidene difluoride membranes using a Mini Blot Module (Thermo Fisher Scientific K.K.) at 20 V for 60 min. After blocking with 5% bovine serum albumin in Tris-buffered saline with Tween-20, the membranes were incubated overnight with primary antibodies (anti-*FTL* antibody [D-9, 1:500, mouse monoclonal, sc-74513; Santa Cruz Biotechnology), anti-*GAPDH* antibody (1:5,000, rabbit monoclonal, sc-25778; Santa Cruz Biotechnology), and then incubated with secondary antibody (1:2,000, anti-mouse IgG, HRP-linked antibody 7076P2; Cell Signaling Technology, Tokyo, Japan); (1:5,000, anti-rabbit IgG, HRP-linked antibody 7074S, Cell Signaling Technology)] for 45 min. Membranes were then stained with ImmunoStar LD (Wako Pure Chemical Industries), and chemiluminescence signals were detected using a C-DiGit Blot Scanner (LI-COR Biosciences, Lincoln, NE, USA). In addition to *FTL* and *GAPDH*, we investigated the expression of p21, p27, CDK2, and pRb. The antibodies used are listed in Table I. After first blotting *GAPDH* to confirm that the recovered proteins were correctly quantified, the other proteins were tested under the same conditions (except for the primary and secondary antibody concentrations) on different membranes.

Cell morphology. The morphology of the two mesothelioma cell lines was observed after NC/*FTL* siRNA transfection at 0, 24, 48, and 72 h using a CKX53 microscope equipped with a DP21 digital camera (Olympus).

Cell proliferation assay. The two mesothelioma cell lines (1x10⁴) were transfected with 1 pmol *FTL* siRNA or NC siRNA in 96-well plates. The proliferation rates were determined at 48 and 72 h by quantifying ATP using the Cell Titer-Glo 2.0 reagent and GloMax Explorer microplate reader (Promega), according to the manufacturer's protocols.

Cell cycle assay. The two mesothelioma cell lines (5x10⁴) were transfected with 10 pmol *FTL* or NC siRNA in 24-well plates for 72 h. Then, the cells were collected and fixed in ice-cold 70% ethanol in 15 ml centrifuge tubes for approximately 1 h. After ethanol removal, the Guava Cell Cycle Reagent (Luminex) containing propidium iodide was used to determine the number of cells at different stages of the cell cycle by labeling the cellular DNA. The labeling signal intensity was measured using a Guava EasyCyte Mini flow cytometer (Guava Technologies) according to the manufacturer's protocol.

Cell invasion assay. The two mesothelioma cell lines (3x10⁴) were cultured in BD FluroBlok culture inserts containing 8 µm pores (BD Biosciences) coated with Geltrex Matrigel (Thermo Fisher Scientific, K.K.) after transfection with 10 pmol *FTL* siRNA or NC siRNA according to the manufacturer's protocols. After 48 h, invaded cells were stained with Hoechst 33324 (Thermo Fisher Scientific, K.K.) for 10 min, and the number of cells visualized using an IX81 fluorescent microscope equipped with a DP80 digital camera (Olympus) was counted using CellProfiler cell imaging software (14).

Table I. List of Primary Antibodies.

Primary antibody	Concentration	Lot number	Catalogue number	Type	Source
FTL	1:500	D-9	sc-74513	Mouse monoclonal	SCB
p21	1:2,000	12D1	2947T	Rabbit monoclonal	CST
p27	1:2,000	D69C12	3686S	Rabbit monoclonal	CST
CDK2	1:2,000	78B2	2546T	Rabbit monoclonal	CST
pRb	1:2,000	D20B12	8516S	Rabbit monoclonal	CST
GAPDH	1:5,000	FL-335	sc-25778	Rabbit monoclonal	SCB

FTL, ferritin light chain; pRb, phosphorylated retinoblastoma protein; SCB, Santa Cruz Biotechnology, Inc.; CST, Cell Signaling Technology, Inc.

Cell migration assay. The two mesothelioma cell lines (1×10^4) were cultured in BD FluroBlok culture inserts containing $8 \mu\text{m}$ pores (BD Biosciences) after transfection with 10 pmol FTL siRNA or NC siRNA according to the manufacturer's protocols. After 48 h, the migrated cells were stained with Hoechst 33324 (Thermo Fisher Scientific K.K.) for 10 min, and the number of cells visualized with a fluorescent microscope was counted using CellProfiler cell imaging software (14).

Apoptosis and necrosis assays. The two mesothelioma cell lines (1×10^5) were incubated with the FTL/NC siRNA in 96-well plates for 24 h, and the RealTime Glo Annexin V Apoptosis Assay reagent (Promega) was added to the cells after transfection. Relative levels of apoptosis and necrosis were measured at 0, 3, 6 and 9 h after transfection by analyzing luminescence and fluorescence with a GloMax microplate reader (Promega) according to the manufacturer's recommended protocol.

Statistical analysis. The data are expressed as mean \pm standard deviation (SD) from three independent experiments, and a paired t-test was used to compare the data using Microsoft Excel for Mac (version 16; Microsoft Corporation). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

FTL mRNA expression was upregulated in malignant mesothelioma. The CCLE database showed a higher expression of FTL mRNA in mesothelioma than in other malignant tumors (Fig. 1A). We also searched for the expression of FTL in mesothelioma cell lines by distinguishing subtypes using the DepMap Portal database. Eleven epithelioid mesotheliomas, two sarcomatoid mesotheliomas, and six biphasic mesotheliomas showed relatively high expression among the diverse malignancies (Fig. 1B). In addition, reanalysis of our previously obtained gene expression data of malignant mesothelioma (epithelioid mesothelioma) showed higher expression of FTL mRNA in all six cases, as shown in the line graph (Fig. 1C). In the reanalysis of gene expression data, the six lung adenocarcinomas also showed high FTL mRNA expression.

Transfection of FTL siRNA into mesothelioma cell lines downregulated expression levels of FTL. Compared with

cells transfected with NC siRNA, the expression levels of FTL mRNA, examined by RT-PCR, were suppressed in cells transfected with FTL siRNA by 75.5% (the Cq of the NC-siRNA group was 10.92, 10.97, and 10.97, and that of the FTL-siRNA group was 12.99, 12.92 and 13.03) in ACC-MESO-1 and 98.2% (the Cq of the NC-siRNA group was 10.0, 10.05, and 10.14, and that of the FTL-siRNA group was 15.9, 15.81, and 15.87) in CRL-5915 cells (Fig. 2A), and the expression levels of FTL protein, examined by western blotting, were suppressed by 85% in ACC-MESO-1 and 87% in CRL-5915 cells (Fig. 2B).

Transfection of FTL siRNA did not change morphology of mesothelioma cells. Observation of cell lines at 0, 24, 48, and 72 h after FTL siRNA transfection did not change the morphology of short spindle to pleomorphic cells in ACC-MESO-1 and mainly changed polygonal cells in CRL5915 compared to cells transfected with NC siRNA. The morphology of the FTL siRNA-transfected group appeared to be more pronounced, but this was due to suppressed proliferation and low density (Fig. 2C).

Transfection of FTL siRNA suppressed proliferation of mesothelioma cell lines. Cell lines transfected with FTL siRNA showed significantly reduced proliferation compared to that of NC. The proliferation ability of ACC-MESO-1 cells was reduced by 11.0% at 48 h and 19.5% at 72 h, and that of CRL-5915 cells was reduced by 4.02% at 48 h and 8.96% at 72 h (Fig. 3A).

FTL siRNA-transfection in mesothelioma cell lines increased the number of cells at the G₁ phase. Transfection with FTL siRNA increased the number of ACC-MESO-1 and CRL-5915 cells in the G₁ phase of the cell cycle from 65.6 to 76.4% and from 65.3 to 93.9%, respectively, compared to NC. In contrast, the number of cells in the G₂ and S phases decreased from 14.3 to 8.2% and from 21.7 to 14.0% for ACC-MESO-1 cells, and from 14.1 to 0% and from 20.3 to 10.6% for CRL-5915 cells, respectively (Fig. 3B).

Transfection of FTL siRNA did not affect the ability of invasion, migration, apoptosis, and necrosis of mesothelioma cell lines. Transfection of ACC-MESO-1 and CRL-5915 cell lines with FTL siRNA had no significant effect on their invasion (Fig. 3C),

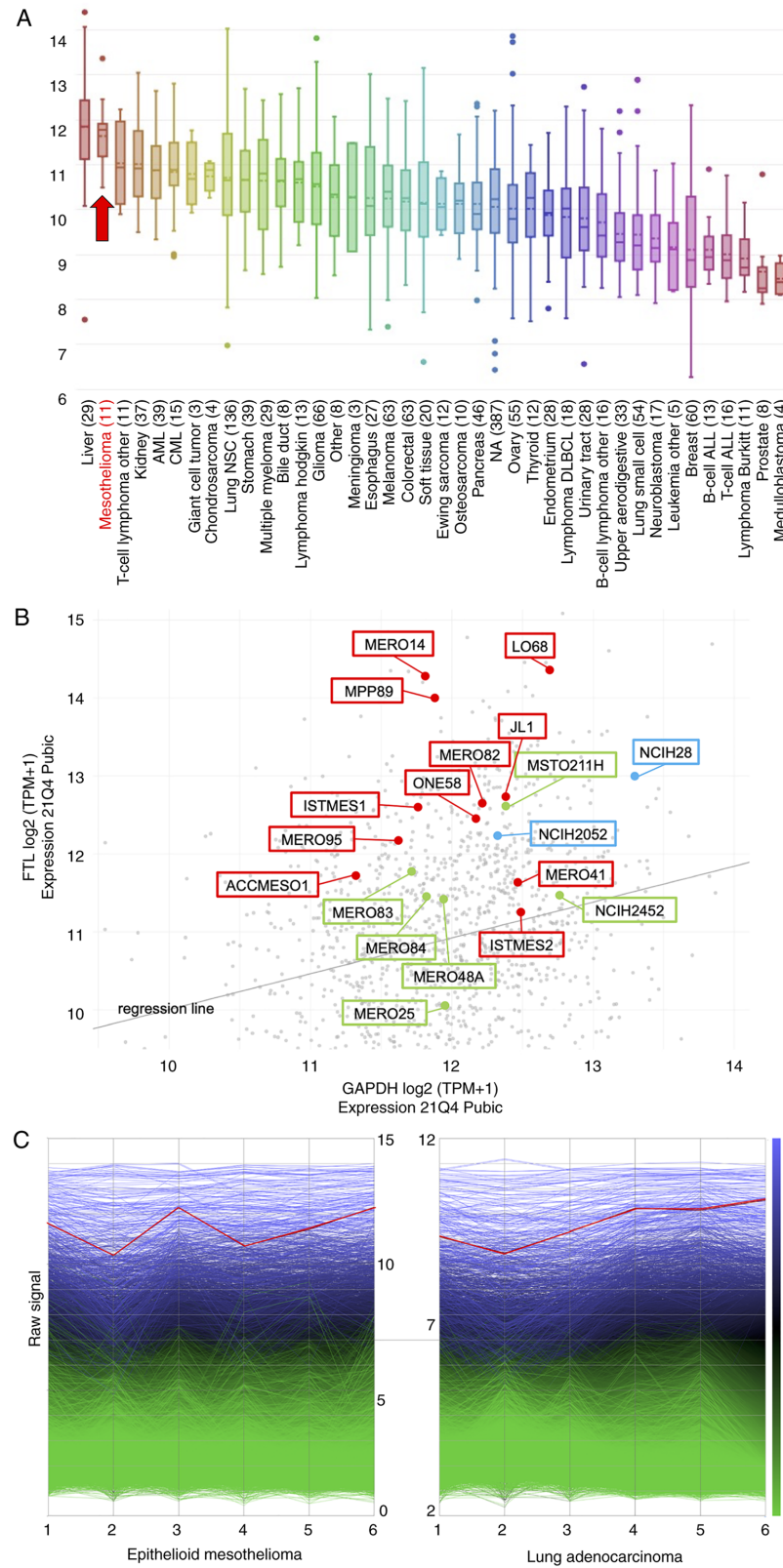


Figure 1. Gene expression levels of FTL among malignant tumors, including mesothelioma. (A) In the online database, Cancer Cell Line Encyclopedia, FTL, indicated by red arrow, shows higher expression in malignant mesothelioma compared with other malignant tumors. (B) DepMap Portal database showed relatively high expression of FTL in three subtypes of malignant mesothelioma; red dots indicate epithelioid, blue dots indicate sarcomatoid and green dots indicate biphasic subtypes, amongst the other malignant tumors, indicated by gray dots. (C) Gene expression analysis in six epithelioid mesotheliomas and six lung adenocarcinomas shows high expression of FTL in both epithelioid mesothelioma and lung adenocarcinoma, indicated by the red line. Other genes are shown as blue or green lines. FTL, ferritin light chain.

migration (Fig. 3D), apoptosis (Fig. 3E), or necrosis ability (Fig. 3F), compared to cells transfected with NC siRNA.

Downregulation of FTL increased expression of p21 and p27 and decreased expression of CDK2 and pRb. Western blotting

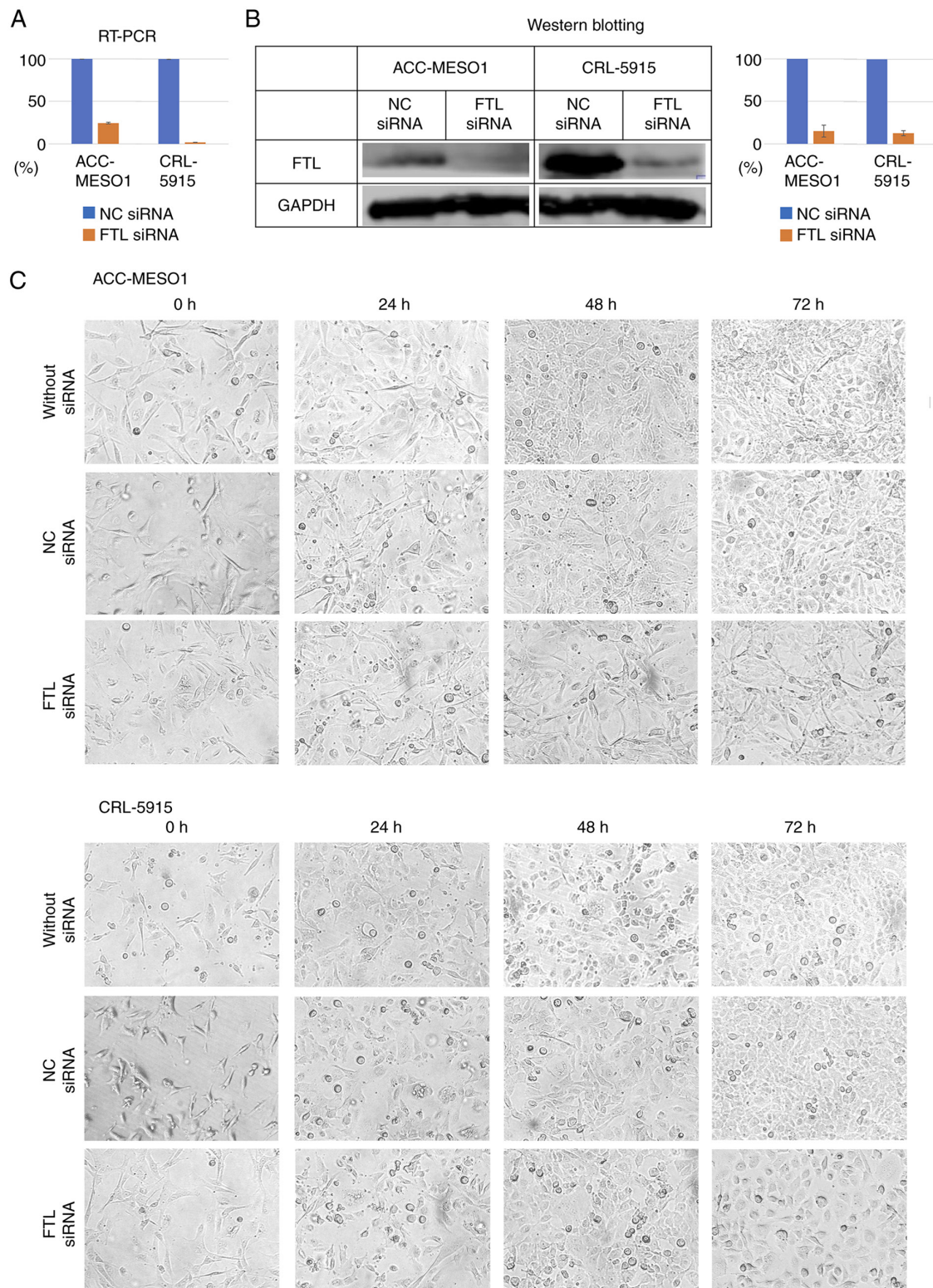


Figure 2. Results of RT-qPCR and western blotting comparing cells transfected with FTL siRNA and NC in two mesothelioma cell lines. (A) Transfection of FTL siRNA suppressed the expression levels of FTL mRNA (as examined by RT-qPCR). (B) Western blotting indicated that the expression level of the FTL protein of FTL siRNA transfected cell lines was significantly suppressed in ACC-MESO-1 and in CRL-5915 cells. The graph shows the relative expression levels of FTL mRNA in the FTL siRNA-transfected cell lines compared with NC. (C) Images show morphology of two mesothelioma cell lines at 0, 24, 48 and 72 h after siRNA transfection. No significant morphological changes were observed among cells without siRNA transfection, NC siRNA transfection or FTL siRNA transfection. FTL, ferritin light chain; siRNA, short interfering RNA; NC, negative control; RT-qPCR, reverse transcription-quantitative PCR.

showed an increase in the expression levels of p21 and p27 and decreased levels of CDK2 and pRb in FTL siRNA-transfected mesothelioma cell lines compared to cells transfected with

NC siRNA. For ACC-MESO-1/CRL-5915, p21 increased by 113/37%, p27 increased by 148/74%, CDK2 decreased by 48/60%, and pRb decreased by 29/50% (Fig. 4).

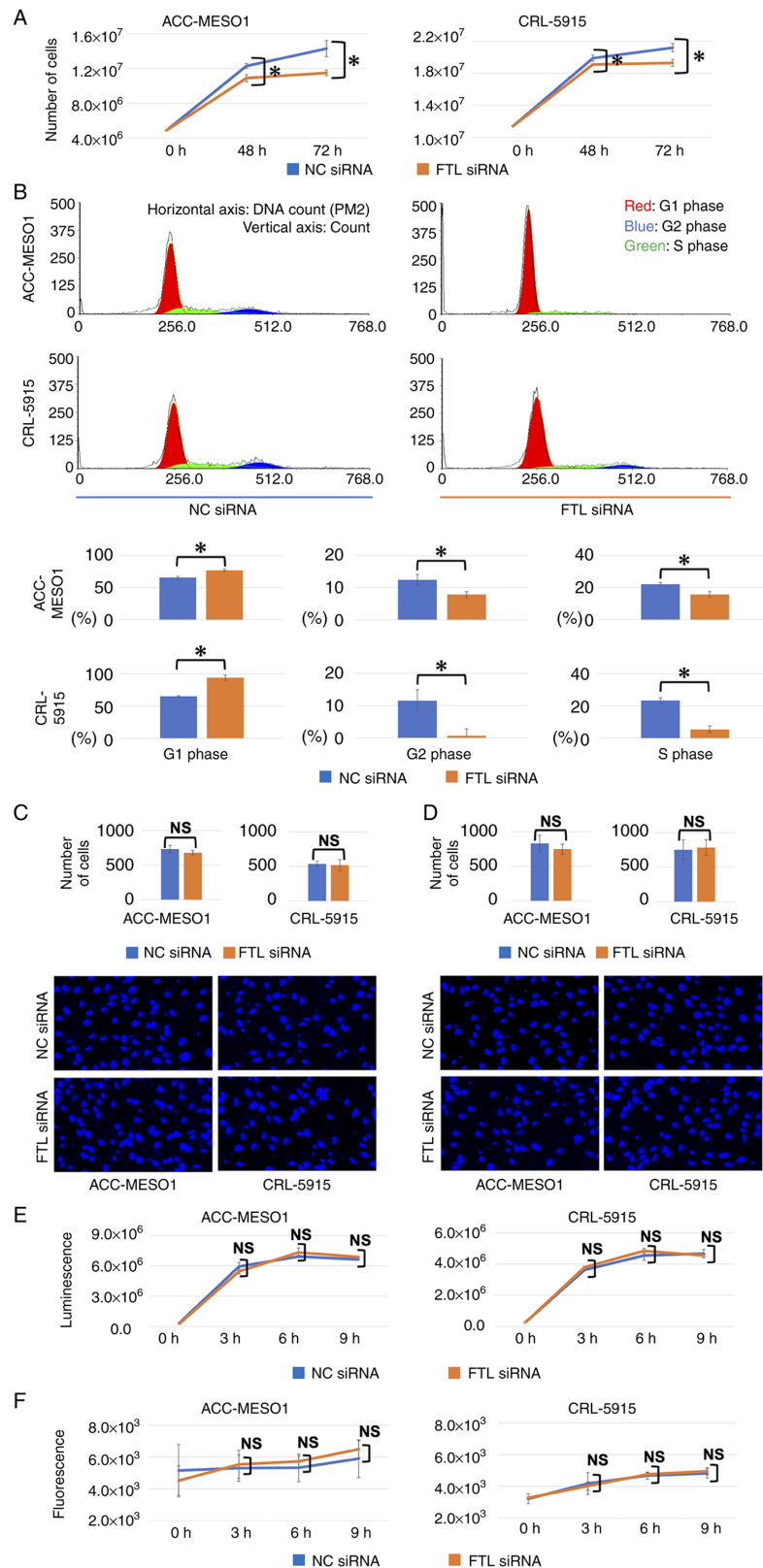


Figure 3. Results of the proliferation assay, cell cycle assay, invasion assay and migration assay comparing cells transfected with FTL siRNA and NC in two mesothelioma cell lines. (A) FTL downregulation significantly reduced proliferation of mesothelioma cell lines compared with NC siRNA-transfected cells. The vertical axis indicates number of cells. (B) By downregulation of FTL, the number of cells in G₁ phase of the cell cycle increased from 65.6 to 76.4% in ACC-MESO-1 cells and from 65.3 to 93.9% in CRL-5915 cells compared with cells transfected with NC siRNA. The vertical axis indicates the count of cells and the horizontal axis indicates DNA count (PM2) in the upper four figures and the percentage of cells at each phase in the lower six figures. (C) Downregulation of FTL does not affect the invasion ability of mesothelioma cell lines. In the graph, the vertical axis indicates the number of cells. Representative fluorescent microscopy images from each group are shown (magnification, x200). (D) Downregulation of FTL did not affect ability of migration of mesothelioma cell lines. In the graph, the vertical axis indicates the number of cells. Representative fluorescent microscopy images from each group are shown (magnification, x200). (E) Downregulation of FTL does not affect the apoptosis of mesothelioma cell lines. The vertical axis indicates the luminescence level. (F) Downregulation of FTL does not affect the necrosis of mesothelioma cell lines. The vertical axis indicates the fluorescence level. *P<0.05. FTL, ferritin light chain; siRNA, short interfering RNA; NC, negative control.

	ACC-MESO1		CRL-5915	
	NC siRNA	FTL siRNA	NC siRNA	FTL siRNA
FTL				
p21				
p27				
CDK2				
pRb				
GAPDH				

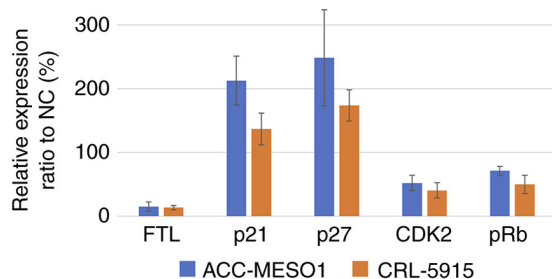


Figure 4. Western blotting of extracts from FTL siRNA transfected cell lines indicates suppression of the expression levels of FTL compared with cells transfected with NC siRNA, increased expression levels of p21 and p27 and decreased levels of CDK2 and pRb. The graph shows the relative expression levels of each protein in the FTL siRNA-transfected cell line compared with NC. FTL, ferritin light chain; pRb, phosphorylated retinoblastoma protein; NC, negative control; siRNA, short interfering RNA.

Discussion

Malignant mesothelioma is a highly aggressive tumor with extremely poor prognosis, and its incidence is increasing worldwide (1), but the molecular mechanism of carcinogenesis and progression of malignant mesothelioma remains unclear, and effective therapy has not been established. Pleurectomy/decortication (P/D) or extra-pleural pneumonectomy (EPP), selected as surgical therapy for malignant pleural mesothelial patients, improves patients' overall survival (15,16). P/D and EPP aim for macroscopic complete resection of the tumor, but microscopic complete resection is almost impossible because malignant mesothelioma occurs in the pleura and anatomically, there is no margin between the tumor and stromal tissue. Radiation therapy has not been fully investigated because of the small number of studies (4). A platinum-pemetrexed combination is the standard for first-line chemotherapy; however, no standard second-line treatment has been discovered. To determine the optimal treatment for individual patients, cytotoxic agents, targeted therapy, and immunotherapy are under investigation (5). Recently, a study showed improvement in the overall survival of malignant mesothelioma patients treated with nivolumab plus ipilimumab (17). Despite these developments, an ideal treatment protocol for malignant mesothelioma is not yet available, and a more detailed understanding of this disease is required.

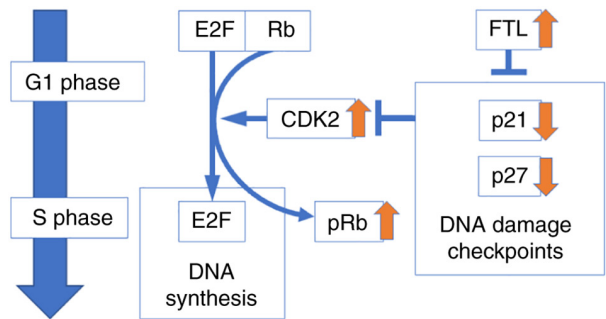


Figure 5. Schematic diagram of the effect of FTL on the cell cycle. FTL may have increased proliferative capacity of malignant mesothelioma by suppressing DNA checkpoint associated genes, p21 and p27, inducing activation of CDK2, which inactivated Rb by phosphorylation into pRb and activated E2F to promote the cell cycle. Orange arrows indicate the estimated status of each gene in malignant mesothelioma, blue arrows indicate the activation of the pathway and blue bars indicate suppression of the pathway. FTL, ferritin light chain; E2F, early 2 factor; RB, retinoblastoma protein; p-, phosphorylated.

Ferritin is a protein involved in iron metabolism that functions intracellularly or extracellularly and contributes to proliferation, angiogenesis, immunosuppression, and iron delivery, not only in non-tumor cells but also in tumor cells. Ferritin consists of two subunits, light chain (L-ferritin, FTL) and heavy chain (H-ferritin), which are functionally and genetically distinct. H-ferritin possesses enzymatic activity and can oxidize ferrous iron to ferric iron. L-ferritin lacks enzymatic activity and thus does not contribute to iron oxidation and uptake; however, L-ferritin has a higher number of carboxyl groups lining the ferritin cavity, which serve as iron nucleation sites that mineralize iron faster. Moreover, the L-ferritin monomer contains a salt bridge within its helical fold, which confers greater stability to the ferritin complex (3). Recently, high expression of FTL protein or mRNA has been associated with tumor malignancy. In colorectal cancer, FTL is upregulated and promotes resistance to 5-fluoro-uracil, migration, invasion, and metastasis of tumor cells (7). High expression of FTL protein in gastric cancer patients has been proportionally associated with the depth of tumor invasion, differentiation grade, lymph node metastasis, and TNM stage, and inversely associated with recurrence-free survival and overall survival (8). The expression of FTL is higher in glioblastoma than in low-grade gliomas. FTL colocalizes with GADD45A in the nucleus of glioblastoma cells and regulates the GADD45A/JNK pathway, which contributes to tumor cell growth (9). Previous studies have reported the association of ferritin, including L-ferritin and H-ferritin, with tumor malignancy (3), the possibility that FTL and FTH are involved in mesothelioma in relation to iron metabolism (18,19), and Mohr *et al* reported 302 upregulated genes, including FTL, and 160 downregulated genes in epithelioid mesothelioma cells of *ex vivo* resected specimens compared to mesothelial cells by microarray gene expression analysis (11), but the overall molecular mechanism is still unclear.

The CCLE database showed relatively high expression of FTL in mesothelioma compared to other malignant tumors, and reanalysis of our previous microarray data at the gene level showed high FTL expression in epithelioid mesotheliomas. We

then investigated the function of FTL in malignant mesothelioma *in vitro*. Downregulation of FTL mRNA using siRNA induced a decrease in proliferation of mesothelioma cell lines compared to that in cells transfected with NC siRNA. Furthermore, mesothelioma cells were arrested in the G¹ phase: the number of cells in the G¹ phase increased and the number of cells in the S and G₂ phases decreased. In contrast, FTL downregulation did not affect cell morphology, migration, invasion, apoptosis, or necrosis. These results indicate that FTL influences mesothelioma growth purely by promoting the cell cycle; therefore, we examined the relationship between FTL and cell cycle-related genes. CDK2 has a considerably broad substrate profile and phosphorylates a large number of proteins involved in cell cycle progression (e.g., p27KIP1 and RB), DNA replication, histone synthesis, and centrosome duplication (20–22). Much of the control over CDK2 involves the synthesis and availability of cyclins. RB and E2F regulate the expression of CDK2, cyclin E1, and cyclin E2 transcripts and proteins (23–27). Recently, it has become clear that deregulation of CDK2 also occurs frequently in certain types of cancer (28). The p21CIP1 acts as a DNA damage checkpoint, which is a critical downstream target gene of p53 that inhibits DNA synthesis, whereas p27KIP1 is responsive to mitogenic signaling as a further control of deregulated proliferation (29,30). Western blotting analysis showed higher expression of p21 and p27, and lower expression of CDK2 and pRb in mesothelioma cell lines transfected with FTL siRNA compared to those transfected with NC siRNA. This suggests that FTL may downregulate p21 and p27 and upregulate CDK2, which induces phosphorylation of Rb, promoting the cell cycle at the G¹ phase in malignant mesothelioma cells. There was no difference in cyclin E1 expression between cell lines transfected with FTL siRNA and NC siRNA (data not shown). Although these are the results of experiments using epithelioid mesothelioma cell lines, the function of FTL in sarcomatoid and biphasic mesothelioma is worth examining because the expression of the *FTL* gene was relatively higher in sarcomatoid and biphasic mesothelioma than in other tumors in the DepMap Portal. In addition, *in vivo* experiments will be conducted to determine whether FTL functions in malignant mesothelioma *in vivo* as it does *in vitro*.

From these results, we concluded that FTL increases proliferation in malignant mesothelioma by suppressing DNA checkpoint-related genes, *p21* and *p27*, inducing activation of CDK2 and inactivation of Rb, which inactivates E2F and promotes the cell cycle (Fig. 5). Therefore, FTL is expected to be a new therapeutic target or diagnostic marker for malignant mesothelioma. Further investigation of the biological mechanism of FTL in malignant mesothelioma is required.

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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

TK, VJA and YT designed the study. VJA and YT supervised and facilitated the study. TK, KK, YF and IE performed the experiments. TK and VJA analyzed the data and wrote the manuscript. TK and VJA interpreted the results. VJA and YT confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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