Research Article

LIMS2 is Downregulated in Osteosarcoma and Inhibits Cell Growth and Migration

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Background/objective. LIM and LIM zinc finger domain containing 2 (LIMS2) is one of the two members of LIMS family, which plays crucial roles in regulating cell-extracellular matrix adhesion and cell motility. Here, we explored the expression and methylation levels of LIMS2 in osteosarcoma (OS) and the role of LIMS2 in OS progression.

Methods. GEO, GEPIA, and UALCAN databases were used to assess LIMS2 expression in OS. UALCAN and CCLE databases were applied to assess the methylation levels of LIMS2 in OS tissues and cells, which was verified in OS cells using the methylation specific PCR. The effects of LIMS2 on regulating OS cell growth, migration and invasion were determined by CCK-8, Edu staining, and transwell chambers, respectively. The role of LIMS2 in the activation of MAPK signaling was assessed using western blotting assay in OS cells.

Results. LIMS2 expression was declined in OS tissues and cells, while its methylation level was increased. The low expression of LIMS2 was associated with shorter overall survival and disease-free survival. Overexpression of LIMS2 inhibited cell growth, migration, and invasion and decreased the levels of p-ERK/ERK, p-P38/P38, and p-JNK/JNK.

Conclusion. LIMS2 expression was decreased in OS, which was associated with hypermethylation level and poor prognosis. LIMS2 overexpression inhibited OS cell growth and migration, which may be caused by the suppression of MAPK signaling.

1. Introduction

Osteosarcoma (OS), which mostly affects adolescents, is the most frequently detected primary bone malignant tumor [1, 2]. The 5-year survival rate of low-grade OS is >70%, but drops significantly to below 20% in patients with high-grade OS, which is characterized by early metastasis and high recurrence rate [3, 4]. Unfortunately, about 20% of OS are diagnosed with metastasis at first diagnosis, resulting in poor response rate and prognosis [1]. Thus, it is of great significance to further reveal the mechanisms underlying the progression of OS.

LIMS family consisting of two members, LIMS1 (also known as PINCH-1) and LIMS2 (also known as PINCH-2), plays crucial roles in regulation cell-extracellular matrix adhesion and movement [5–7]. LIMS1 and LIMS2 share 92% sequence homology and compete for binding to the ankyrin repeat domain of ILK with similar affinities [8]. Like LIMS1, studies have shown that LIMS2 takes part in cancer migration and invasion [9–11]. The expression of LIMS2 was decreased in gastric cancer, which was significantly associated with the increased CpG island methylation. In addition, silencing of LIMS2 promoted the proliferation and migration of gastric cancer cells [12]. Moreover, LIMS2 expression was declined in colon cancer, and LIMS2 overexpression could inhibit the migration of colon cancer cells [11]. However, the role of LIMS2 in the progression of other types of cancers, such as OS remains unknown.
In this study, we analyzed LIMS2 expressions in OS using GEO and TCGA databases, and the results revealed that LIMS2 expression was decreased in OS biopsy samples. In addition, UALCAN and CCLE databases revealed that the methylation level of LIMS2 promoter in OS tissues and cells were increased. All these findings suggested that LIMS2 may play a role in the progression of OS. To this end, we conducted this study to explore LIMS2 expression in OS and to reveal its role in the progression of OS and its potential mechanisms.

2. Materials and Methods

2.1. GEO Datasets and Identification of DEGs. The raw RNA transcriptome dataset (GSE42352) containing the expression data of 84 high-grade OS biopsy samples and 13 normal tissue samples was obtained from the Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) database. The mRNA expression profiling was assessed with the chip-based platform GPL10295 Illumina human-6 v2.0 expression beadchip with nIDs as an identifier. The DEGs (differently expressed genes) between OS tissues and normal tissues were screened using the R software version 4.1.3 (http://www.R project. Org/) [13, 14]. Background correction, standardization, and the calculation of expression values were carried out using package Affy, Impute, and Limma of R software. The limma package was applied to normalize the median value of all samples. After that, a robust multichip average (RMA) was created, and the raw data were log-transformed. Once the log 2 fold change (FC) > 1, the genes were identified as DEGs. Pheatmap and ggplot2 in R software were applied to build the heat map and Volcano plot, respectively [15].

2.2. GEPIA, UALCAN, and CCLE Databases. GEPIA (http://gepia.cancer-pku.cn/index.html) was used to assess LIMS2 expression and its association with the overall survival in OS; UALCAN database (http://ualcan.path.uab.edu/) was used to evaluate the expression methylation levels of LIMS2 in OS, as well as predict the genes correlated to LIMS2; CCLE database (https://portals.broadinstitute.org/ccle/) was also applied to analyze the methylation level of LIMS2.

2.3. Functional Enrichment. R software was applied to assess the enriched pathways of LIMS2 and its associated genes identified from the UALCAN database with a Pearson – CC ≥ 0.4, including Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. Three modules, biological processes (BP), cellular component (CC), and molecular function (MF), were included in the GO analysis. p adjust value < 0.05 was thought as statistically significant.

2.4. Cell Culture. U-2OS, MG-63, Saos-2 and MNNG/HOS, 4 human OS cell lines, and one human normal osteoblast cell line hFOB 1.19 were obtained from American Type Culture Collection (Manassas, VA, USA). Another lung cancer cell line PC-9 was obtained from BeNa Culture Collection (Beijing, China). U-2OS and Saos-2 cells were cultured in McCoy’s 5a Medium, while MG-63 and MNNG/HOS cells were grown in Eagle’s Minimum Essential Medium, all with the supplementation of 10% FBS (Fetal Bovine Serum) and 1% (v/v) penicillin/streptomycin. hFOB 1.19 cells were maintained in 1:1 mixture of Ham’s F12 Medium and Dulbecco’s Modified Eagle’s Medium, supplemented with 2.5 mM L-glutamine, 0.3 mg/ml G418, and 10% FBS. All cell lines were placed at 37°C with 5% CO₂. Cell culture mediums were purchased from Thermo Fisher Scientific (MA, USA).

2.5. Upregulation of LIMS2 Expression. Cells were transfected with the overexpressed plasmid to overexpress LIMS2 and the negative control vector (NC) (cat no. RC229173, Beijing, China) with the help of lipofectamine 2000 (Thermo) according to the manufacture’s descriptions.

2.6. Methylation-Specific PCR (MS-PCR). Genomic DNA (gDNA) was extracted with a QIAamp DNA Mini Kit (Qiagen, Germany) and submitted to sodium bisulfite modification with DNA Methylation Detection Kit (BioChain, USA) in the light of the manufacturer’s descriptions. Then, PCR was carried out using the modified DNA in reaction system of 25 μL with the following conditions: 35 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s. PCR products were separated in 3% agarose gel supplemented with ethidium bromide and the DNA blots were visualized under UV illumination. Unmethylation-specific primers: forward-5’-GGTTGATT TTTAGATTGATGTA-3’, reverse-5’-AACAAATAAAAA TAAACAAAAACAAA-3’;

methylation-specific primers: forward-5’-TGGTTGGA TTTTTAGATTGATGAC-3’, reverse-5’-AACGATAAAAA TAAACGAAAAACAA-3’.

2.7. Quantitative Reverse Transcription-PCR (qRT-PCR). Total RNA samples were extracted using TRIzol reagent (Invitrogen, USA). The RNAs were then reverse transcribed into cDNA using PrimeScript RT Master Mix Kit (RR036A; Takara) in accordance with the descriptions. Next, the PCRs detection was performed using 2× SYBR Green PCR Mastermix (Solarbio, Beijing, China) in a 7500 Real-Time PCR System (Applied Biosystems, USA). Primers applied are shown in Table 1.

2.8. Western Blotting. Total protein was isolated with the RIPA lysis buffer (Solarbio, Beijing, China) and added with 1% protease inhibitor (Solarbio) from cells. Subsequently, same amount

| Table 1: Primer sequences. |
|---------------------------|
| Gene          | Sense (5’-3’)                      | Antisense (5’-3’)                     |
| LIMS2         | GAGGGGCTTCTGGGCTTTTT               | GTACAGCTCCCCCCATTTGCTGT              |
| β-Actin       | TGGAACGGTGAAGGTGACAG               | CGCATCTCATATTTGGAAATGACT             |
Figure 1: Continued.
of proteins (about 20 μg) from each group were separated by 10% SDS-polyacrylamide gels and transferred onto the polyvinylidene difluoride membranes (PVDF; Millipore, Billerica, MA, USA). After that, the membranes were blocked with 5% non-fat milk at room temperature for 60 min to prevent the nonspecific bindings, followed by primary antibody incubation at 4 °C for overnight, including anti-β-actin antibody (cat no. ab8226, Abcam, MA, USA; 1: 5000 dilution), anti-LIMS2 antibody (cat no. ab272666, Abcam; 1: 2000 dilution), anti-p-ERK (cat no. 4370, CST; 1: 2000 dilution), anti-ERK (cat no. 4695, CST; 1: 1000 dilution), anti-p-P38 (cat no. 4511, CST; 1: 1000 dilution), anti-P38 (cat no. 8690, CST; 1: 1000 dilution), anti-p-JNK (cat no. 9251, CST; 1: 1000 dilution), and anti-JNK (cat no. 9252, CST; 1: 1000 dilution) antibodies. After that, the membranes were probed with HRP-conjugated secondary antibodies at room temperature for 1 hour. ProBlot-48 (Tecan, Switzerland) was applied to evaluate protein signaling following immersing in ECL reagent (Millipore, USA). ImageJ software was used for protein quantification.

2.9. CCK-8 (Cell Counting Kit-8) Assay. Cells were placed in 96-well plates with 4,000 cells in each well. For cell growth assessment, cells were cultured with 10% (v/v) CCK-8 solution (Beyotime, Beijing, China) for 4 hours at 37 °C. Then, the OD values (450 nm) were detected with a Spectrophotometer (Fisherbrand™ accuSkran™ GO UV/Vis, Thermo).

2.10. Edu (5-Ethynyl-2′-Deoxyuridine) Staining. Edu staining was performed to assess cell proliferation using the EdU Assay/EdU Staining Proliferation Kit (cat no. ab222421, Abcam). Each well of the 24-well plate 6 × 10^4 cells were plated into each well of the 24-well plate and then transfected with indicated plasmids. After 48 hours, the cells were cultured with 50 μM EdU reagent for 2 hours and fixed with 4% formaldehyde for 0.5 hour, followed by incubation with glycine (2 mg/mL) for 0.5 hour to permeabilize. Next, the cells were incubated with Hoechst 33342 for nuclear staining. The percentage of Edu positive cells was assessed under a fluorescence microscopy (Olympus IX73, Japan).

2.11. Transwell Chamber Assay. Transwell chambers (pore size, 8 μm; BD Biosciences) were applied to detect the effect of LIMS2 on cell migration and invasion capacities. To detect cell migration, 5 × 10^4 cells were seeded into the upper chamber, while 0.60 ml of cell culture medium containing 15% FBS were added into the lower chamber. Following incubation at 37 °C for 24 hours, the cells on the upper side of the filters were removed with cotton swabs, while cells below the filters were first fixed with methanol for 15 min and then stained with 0.1% crystal violet. To detect cell invasion, the transwell chambers precoated with Matrigel were used and proceed as described as the migration assay. The number of migrated and invaded cells was counted under the microscope.

2.12. Statistical Analysis. Each experiment was repeated for three independent times in the current study. SPSS21.0 software (IBM, Armonk, NY, USA) was applied for the statistical analysis with student’s t-test or one-way ANOVA with Tukey’s tests. The p value less than 0.05 was considered a statistical significance.
Expression\textsuperscript{-log2}(TPM+1)

(a)

Expression of LIMS2 in SARC based on sample types

(b)

Expression of LIMS2 in SARC based on patient's race

(c)

Promoter methylation level of LIMS2 in SARC

(d)

Figure 2: Continued.
3. Results

3.1. Bioinformatics Analysis Showed that LIMS2 Expression Was Downregulated While Its Methylation Level Was Increased in OS. To reveal the mechanisms underlying the progression of OS, first, the transcription data of 84 OS tissues and 13 normal tissues were downloaded from the GEO database to identify the DEGs. Figure 1(a) was the correction diagram of removing batch. The PCA (principal component analysis) showed that the tumor group and normal group could be well distanced (Figure 1(b)). Moreover, we observed a good correlation between groups and genetic characteristics (Figure 1(c)). A total of 429 upregulated genes and 418 downregulated genes (including LIMS2) were found between tumor and normal groups, as shown by the volcano plot (Figure 1(d)). These results indicated that LIMS2 was downregulated in OS.

To further explore the expression of LIMS2 in OS, we recruited the GEPIA and UALCAN database. We observed that the expression of LIMS2 was decreased in many kinds of cancers, including sarcoma (SARC) (Figures 2(a)–2(c)) regardless of race, gender, and age (Figure 2(b)). In addition, the promoter methylation level of LIMS2 was significantly increased in sarcoma compared to normal group, as shown in the UALCAN database (Figure 2(d)). Consistently, the CpG island methylation level of LIMS2 showed a high level in OS cell lines (Figure 2(e)). Moreover, the low expression level of LIMS2 was linked to lower overall survival rate and lower disease-free survival rate in OS (Figures 3(a) and 3(b)). These results further revealed a lower expression pattern of LIMS2 in OS, which was accompanied by high methylation level and related to poor prognosis.

3.2. LIMS2-Related Genes Were Enriched in MAPK Signaling Pathway. Then, we assessed the enriched pathways involved LIMS2 and its related genes identified by the UALCAN database. The GO analysis showed that the genes were enriched in muscle system process, cell-substrate junction, cell...

Figure 2: Bioinformatics analysis of the expression and methylation levels of LIMS2 in OS. (a) LIMS2 expressions in different kinds of cancers were assessed using the GEPIA database. (b) LIMS2 expression in sarcoma was assessed by UALCAN database. (c) LIMS2 expression in normal and tumor tissues was evaluated from the GEO (GSE42352). (d) The methylation levels of LIMS2 in sarcoma tissues and normal tissues were analyzed using the ualcan database. (e) CCLE database was applied to assess the methylation levels of LIMS2 in OS cells.

Figure 3: Low expression of LIMS2 was linked to poor prognosis in OS. The relationships between LIMS2 expression levels and (a) the overall survival and (b) the disease-free survival rates of patients with OS were evaluated using the GEPIA database.
Figure 4: Continued.
adhesion, actin binding, and cadherin binding pathways (Figure 4(a)). KEGG analysis showed that the genes were mainly enriched in focal adhesion, tight junction, MAPK signaling pathway, and adherens junction (Figure 4(b)). These results indicated that LIMS2-related genes may play a role in regulating cell motility.

3.3. LIMS2 Expression Was Downregulated in OS Cells. Next, we assessed LIMS2 expression and methylation levels in OS tissues. Compared with the expression level of LIMS2 in normal osteoblast cell line hFOB 1.19, both the mRNA (Figure 5(a)) and protein (Figures 5(b) and 5(c)) levels of LIMS2 were decreased in OS cell lines (U-2OS, MG-63, Saos-2, and MNNG/HOS). In contrast, LIMS2 methylation level was increased in OS cell lines compared with hFOB 1.19 cells (Figure 5(d)). These results verified LIMS2 level was declined in OS.

3.4. LIMS2 Inhibited OS Cell Growth and Migration. Additionally, we assessed the role of LIMS2 in OS progression in vitro. LIMS2 expression was remarkable increased in U-2OS and Saos-2 cells following the cell transfection with LIMS2 plasmid (Figure 6(a)). In comparison with the control group, cell growth was significantly suppressed when LIMS2 expression was upregulated, as determined by the CCK-8 assay (Figure 6(b)) and Edu staining (Figure 6(c)). In addition, LIMS2 overexpression caused significant inhibition in cell migration (Figure 6(d)) and invasion.
These results demonstrated that LIMS2 overexpression could suppress cell growth and migration in OS.

3.5. LIMS2 Overexpression Inhibited the Activation of MAPK Signaling in OS Cells. Since the LIMS2 and its associated genes were enriched in the MAPK signaling pathway, we assessed the effects of LIMS2 on the activation of MAPK signaling in vitro. The results demonstrated that LIMS2 overexpression significantly decreased the levels of p-ERK/ERK, p-P38/P38, and p-JNK/JNK in U-2OS and Saos-2 cell lines (Figure 7). These results confirmed that LIMS2 overexpression could repress the activation of MAPK signaling in OS.

4. Discussion

Bioinformatics databases have shown that LIMS2 expression was decreased in OS tissues, indicating that LIMS2 may be involved in OS progression. In the current study, we first explored LIMS2 role in the motility of OS. The results verified a downregulated expression of LIMS2 in OS, while its methylation level was increased, and overexpression of LIMS2 caused significant suppressions of cell growth and migration abilities in OS.

Currently, evidence has demonstrated that LIMS2 is implicated in the carcinogenesis of several kinds of cancers. For example, Kim et al. [12] reported that hypermethylation induced silencing of LIMS2 was observed in majority of the gastric cancer cell lines and about half of primary gastric tumors and silencing of LIMS2 promoted the viability and migration of gastric cancer cells. LIMS2 expression was declined in colon cancer, and overexpression of LIMS2 significantly inhibited the migration of colon cancer cells [11]. In addition, LIMS2 was highly expressed in melanoma cells with heparinase gene silencing (HPSE), leading to cell apoptosis [16]. Consistently, it has been shown by the online database that LIMS2 expression was decreased in OS, which was then verified in OS cells using the western blotting assay. Moreover, the low expression of LIMS2 was related to lower overall survival and disease-free survival rates of patients with OS. Interestingly, we found that the methylation level at the promoter of LIMS2 gene was increased in OS cells compared with the normal osteoblast, which was consistent with the finding in gastric cancer [12]. However, LIMS2 mRNA level was increased in malignant mesothelioma compared with carcinomas involving serosal cavities [17], with its function in the progression of malignant mesothelioma.
Figure 6: Continued.
remaining unknown. Different cancer types may cause this expression difference. Moreover, the in vitro assay showed that LIMS2 overexpression inhibited the growth, migration, and invasion of OS cells, suggesting that LIMS2 functioned as a tumor suppressor in OS, which was similar as reported in gastric cancer [12] and colon cancer [11].

The MAPK signaling exerts an important role in the regulation of the progression of OS [18–20]. Here, the pathway enrichment analysis showed that LIMS2 and its correlated genes were mainly enriched in the MAPK signaling. Western blotting assay results showed that LIMS2 overexpression led to significant inhibitions in the levels of p-ERK, p-P38, and p-JNK, further suggesting that the MAPK signaling may be a downstream pathway through which LIMS2 inhibited the progression of OS. Chen et al. [21] reported that LIMSI regulated the ERK-Bim pathway and triggered

Figure 6: LIMS2 inhibited OS cell growth and migration. U-2OS and Saos-2 cells divided into control, NC and LIMS2 groups were collected for the following assays. (a) LIMS2 protein levels in different groups were determined using western blotting assay. (b) CCK-8 assay and (d) Edu staining were applied for cell growth detection. (d, e) Cell migration and invasion capacities were tested with the transwell chambers (n = 3, ^p < 0.05, ^^p < 0.01, ^^^p < 0.001, and LIMS2 group vs. NC group).

Figure 7: LIMS2 overexpression inhibited the activation of MAPK signaling in OS cells. The protein levels of ERK, p-ERK, P38, p-P38, JNK, and p-JNK in different groups of U-2OS and Saos-2 cells were detected by western blotting assay (n = 3, ^p < 0.05, ^^p < 0.01, LIMS2 group vs. NC group).
apoptosis resistance in cancer cells, indicating a link between PINCH family and MAPK signaling. Montanez et al. [22] demonstrated that deletion of LIMS1 led to a sustained activity of JNK in primitive endoderm (PrE) cells. Here, we first explored LIMS2 effect on the activation of MAPK signaling in cancer cells, and our results demonstrated that overexpression of LIMS2 could significantly inhibit the activation of MAPK signaling. However, whether MAPK signaling is involved in LIMS2-mediated inhibitions of cell growth and migration in OS remains to be further studied.

There are still limitations for the current study. The expression of LIMS2 should be detected in human OS tissues, and its association with patients’ prognosis should also be explored. As mentioned earlier, another limitation is that we did not explore the underlying mechanisms by which LIMS2 inhibits cell growth and migration in OS, such as the MAPK signaling. We intend to explore these in future studies.

In summary, this study demonstrated that LIMS2 expression was decreased in OS, which was associated with hypermethylation level and poor prognosis. LIMS2 overexpression inhibited OS cell proliferation and migration, which may be mediated by the suppression of MAPK signaling. Regents used to upregulate LIMS2 expression, such as the methylation inhibitor, might be a potential treatment method to repress cell migration in OS.

Data Availability
All data generated or analyzed during this study are included in this published article.

Conflicts of Interest
The authors state that there are no conflicts of interest to disclose.

Authors’ Contributions
Chenyeng Su and Xiaona Cai designed the study, completed the experiment, and supervised the data collection, Taotao Xu and Yungang Wu analyzed the data and interpreted the data. Licong Wang, Pinjie Chen, and Chenxian Su prepared the manuscript for publication and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

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