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Long noncoding RNA PVT1 promotes metastasis *via* miR-484 sponging in osteosarcoma cells

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Abstract. – OBJECTIVE: Long noncoding RNAs (lncRNAs) are widely involved in various malignancies including osteosarcoma. In the current study, we aimed to illustrate the role of lncRNA plasmacytoma variant translocation 1 (PVT1) in osteosarcoma.

PATIENTS AND METHODS: Expression of PVT1 and microRNA-486 (miR-486) in osteosarcoma tissue specimens and cell lines were detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) assays and in situ hybridizations (ISH) assay. Transwell migration/invasion assays were performed to determine the metastatic ability changes in osteosarcoma cells. Kaplan-Meier survival analysis was applied to analyze the overall survival (OS) of patients with osteosarcoma. Luciferase assays were used to evaluate the targeted binding effect between PVT1 and miR-486.

RESULTS: We illustrated that lncRNA plasmacytoma variant translocation 1 (PVT1) was upregulated in osteosarcoma, and it was correlated with poor prognosis of patients with osteosarcoma. Furthermore, we found that PVT1, *via* constructed loss of function and gain of function assays, promoted osteosarcoma cells migration and invasion. Meanwhile, we demonstrated that microRNA-486 (miR-486) was involved in PVT1-induced migration and invasion. We also uncovered that miR-486 was downregulated in osteosarcoma tissue specimens and cell lines. Functionally, we showed that upregulation of miR-486 reversed the facilitative effect of PVT1 on osteosarcoma cells migration and invasion, and vice versa. Mechanically, we illustrated that PVT1 interacted with miR-486 in a reciprocal suppressed manner. Moreover, we found that miR-486 could target to PVT1 *via* Luciferase assay. Lastly, we proved that PVT1 promoted osteosarcoma cells migration and invasion through miR-486 sponging.

CONCLUSIONS: We demonstrated that PVT1, functioning as an oncogene, promotes osteosarcoma cells metastasis *via* miR-486 sponging. PVT1/miR-486 axis might be a novel target in the molecular treatment of osteosarcoma.

Key Words:
PVT1, MiR-486, CeRNA, Metastasis, Osteosarcoma.

Introduction

As the most common malignant bone tumor in children and adolescents, the incidence of osteosarcoma is 65 cases per year, representing 5% of all childhood cancers (0.5 cases per 100 000 per year)[1]. Approximately 15-20% of patients with osteosarcoma have already had pulmonary metastases at their first diagnosis, and the prognosis of these patients is depressing, with about less than 20-30% of long-term survivors[2]. Although integrated therapies including surgical resection, chemotherapy, and radiotherapy have achieved great development, the overall survival of patients with distant metastasis is still frustrating[3,4]. Therefore, it is still urgent to find out novel metastatic-related molecules and deeper elucidation of their working mechanisms.

Long non-coding RNAs (lncRNAs), more than 200 nucleotides in length, are a large category of transcriptions with non-protein-coding ability[5]. LncRNAs broadly participate in a variety of malignant tumors, including prostate cancer, gastric cancer, melanoma, breast cancer, lung cancer, and osteosarcoma[6-11]. LncRNA PVT1 is located at human chromosome 8q24.21 and contains 9 exons. PVT1 has been identified as a candidate oncogene and is associated with many types of cancers, including breast and ovarian cancers, acute myeloid leukemia, and Hodgkin lymphoma[12-15]. Zheng et al[16] reported that PVT1 promoted angiogenesis of vascular endothelial cell by targeting miR-26b to activate connective tissue growth factor (CTGF)/angiopoietin 2 (ANGPT2). Shen et al[17] found that PVT1 epigenetically silenced microRNA-195 (miR-195) and modulated epithelial-mesenchymal transition (EMT) and chemoresistance in cervical cancer cells. Up to date, there are not much related researches on PVT1 and osteosarcoma.

The functions of lncRNAs include chromatin modification, transcriptional regulation,

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post-transcriptional regulation, and so on[18,19]. As to the post-transcriptional regulation, the most common working mechanism of lncRNAs is co-working with microRNAs (miRNAs) *via* acting as competitive endogenous RNA (ceRNA) or miRNA sponges[20]. Liu et al[21] found that lncRNA nuclear enriched abundant transcript 1 (NEAT1) was upregulated in ovarian cancer (OC), and facilitated OC cells' metastasis by working as a sponge of miR-382. Wang et al[22] uncovered that lncRNA metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) promoted osteosarcoma metastasis and proliferation *via* miR-144-3p sponging. In the current research, PVT1 was shown to be overexpressed in osteosarcoma and closely correlated with poor outcomes of osteosarcoma. Mechanically, PVT1 was demonstrated to promote osteosarcoma cells metastasis by acting as ceRNA of microRNA-486 (miR-486). Our findings presented a new molecular axis in targeting treatment of osteosarcoma.

Patients and Methods

Patients and Tissue Samples

Forty-eight cases of osteosarcoma tissue samples and paired para-tumor tissue samples were obtained from patients undergoing resection of tumor at The First Hospital of Jilin University. The time span was from January 2009 to November 2018. All samples were histopathologically confirmed and obtained with informed consent. No patient received preoperative local or systemic anticancer treatment. Tumor stage was classified according to the guidelines of the 7th Edition of the American Joint Committee on Cancer (AJCC) Cancer Staging Manual of TNM. The investigation was approved by the Institutional Ethics Committee of The First Hospital of Jilin University.

Cell Culture

A human osteoblast cell line hFOB 1.19 was cultured in Dulbecco's Modified Eagle's Medium (DMEM/F12; Gibco, Rockville, MD, USA). Four human osteosarcoma cell lines- MG-63, U2OS, HOS, and 143B were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco). All medium was supplemented with 10% (v/v) fetal bovine serum (FBS, Sigma-Aldrich, St. Louis, MO, USA), 100 IU/ml penicillin, and 100 μg/ml streptomycin (Baomanbio, Shanghai, China). MG-63, U2OS, HOS, and 143B were incubated at 37°C while hFOB1.19 was incubated at 34°C in a humidified atmosphere containing 5% CO_2.

RNA Extraction and Quantitative Real-Time PCR (qRT-PCR)

All the procedures were carried out as previously described[23]. Total RNAs were isolated by TRIzol reagent (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized by a TaKaRa RNA PCR kit (TaKaRa, Dalian, China) according to the manufacturer's protocol. PCR reactions containing SYBR Premix Ex Taq II (TaKaRa) were followed as per the manufacturer's manual. U6 small nuclear RNA and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used as internal controls. Primer sequences were synthesized by RiboBio Co., Ltd. (RiboBio, Guangzhou, China) as listed in Table I.

Oligonucleotides and Plasmids Transfection

Specific small interfering RNAs targeted PVT1 (siPVT1-1 and siPVT1-2) and scramble small interfering RNA (siSCR) were synthesized by GenePharma Co., Ltd., and so were the PVT1 overexpression plasmids containing wild (PVT1) and mutant (PVT1-mut) miR-486 binding sites (GenePharma, Shanghai, China). The miR-486 mimics and negative control mimics (NC mimic), miR-486 inhibitors and negative control inhibitors (NC inhibitor) were purchased from RiboBio to overexpress or knock down miR-486. All the oligonucleotides and plasmids were transfected into osteosarcoma cells based on different requirements by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocols. The sequences of small interfering RNAs were also listed in Table I.

In Situ Hybridizations (ISH) Assay

The procedures were performed as previously reported[23]. Fresh osteosarcoma sections were permeabilized with 0.3% Triton X-100 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 15 min, and then incubated in a hybridization solution containing special probes (RiboBio, Guangzhou, China) targeted miR-486 supplemented with 1% blocking solution. They should be placed in a humid chamber at 37°C overnight. The next day, the sections were rinsed with 0.1% Tris-Buffered Saline and Tween-20 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) solution in 4×sodium citrate buffer (SSC) for 5 min, 0.1% Tween-20 solution in 2×SSC for 5 min, and 0.1%

Table I. Primer sequences and oligonucleotides used in this research.

Gene	Sequences of primers/oligonucleotides
PVT1 forward	TCTGTTGCTCGTAGCTGCTG
PVT1 reverse	CTATGAGTCACCGGGATTTTG
GAPDH forward	TGTTCGTCATGGGTGTGAAC
GAPDH reverse	ATGGCATGGACTGTGGTCAT
miR-486 forward	GTTTCCTGTACTGAGCTGC
miR-486 reverse	GTGCAGGGTCCGAGGT
U6 forward	CGCTTCGGCAGCACATATACTA
U6 reverse	CGCTTCACGAATTTGCGTGTCA
siPVT1-1	CCATCATGATGGTACTTTA
siPVT1-2	GCAGCTTATTATAGACTTA
miR-486 mimics	TCCTGTACTGAGCTGCCCCGAG
miR-486 inhibitor	CTCGGGGCAGCTCAGTACAGGA

Tween-20 solution in 1×SSC for 5 min at 42°C in the dark. Lastly, the sections were triply washed with 1×phosphate-buffered saline (PBS) for 5 min at room temperature and counterstained by 4,6-diamidino-2-phenylindole (DAPI). All sections were observed and photographed under a microscope (Leica, Wetzlar, Germany).

Transwell Assay

The procedure was carried out as previously described[23]. HOS and 143B cells were seeded in a 6-well plate (Corning, Corning, NY, USA) after designated treatments and incubated for 72 h. The upper chambers were precoated with (for invasion assay) or without (for migration assay) Matrigel (1:20, BD Biosciences, Franklin Lakes, NJ, USA) 2 h before plating the cells. Cells were cultured with serum-free media and seeded into the upper chambers at a concentration of 1×10^5/ml. Culture medium supplemented with 10% FBS was placed in the lower chambers. After incubation for 12 h, the invaded or migrated cells were counted after being permeabilized by methanol for 20 min at room temperature and stained with 0.1% (w/v) crystal violet in a dark room.

Dual-Luciferase Reporter Assay

The procedure was carried out as previously described[23]. Reporter plasmids containing wild and mutant sequence of PVT1 (PVT1-luc-wt and PVT1-luc-mut) were designed and chemically synthesized by GenePharma, respectively. The reporter plasmids were co-transfected with miR-486 mimics or NC mimic and incubated for 48 h, individually. Luciferase activity was measured with Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) according to the manufacturer's protocol.

Statistical Analysis

All data were evaluated by GraphPad Prism V5.0 (GraphPad Software, Inc., La Jolla, CA, USA) software and SPSS 19.0 statistical software (IBM, Armonk, NY, USA). Kaplan-Meier survival curves were applied to analyze the overall survival (OS) of patients with osteosarcoma calculating by log-rank test. Pearson's Chi-squared test was used to analyze the correlation between PVT1 and clinicopathological features of patients with osteosarcoma. Differences between two groups were analyzed by the Student's *t*-test. Means of different groups were compared using one-way analysis of variance test and followed by Bonferroni's test. All data were collected from three independently repeated experiments and expressed as mean ± SD. Differences were considered significant if $*p < 0.05$, $**p < 0.01$, $***p < 0.0001$, individually.

Results

PVT1 is Upregulated and Correlated with Poor Prognosis in Osteosarcoma

To determine the expression level of PVT1 in osteosarcoma, qRT-PCR assays were performed, and the results were shown in Figure 1a-b that PVT1 was mostly (40/48, 83.33%) upregulated in the collected 48 osteosarcoma tissue samples. Meanwhile, upregulated PVT1 was found to be more frequently presented in osteosarcoma tissue samples with lymph-node metastasis and distant metastasis (Figure 1c-d). The clinical value of PVT1 in patients with osteosarcoma was further tested. As shown in Figure 1e, patients with high PVT1 expression had

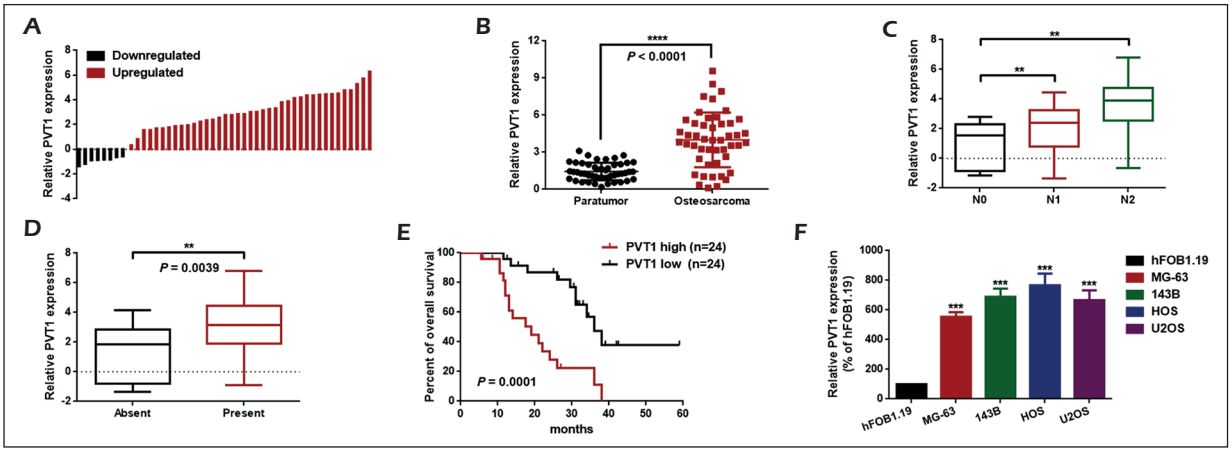

Figure 1. PVT1 is upregulated and correlated with poor prognosis in osteosarcoma. **A**, Expression of PVT1 in collected 48 paired osteosarcoma tissue samples was qualified by a qRT-PCR analysis via log2 ($2^{-\Delta\Delta Ct}$) method. **B**, PVT1 was upregulated in osteosarcoma tissue samples as compared with that in paired paratumor tissue samples. **C**, Expression of PVT1 was remarkably higher in osteosarcoma patients with lymph node metastasis (N1 and N2) than that without lymph node metastasis (N0), determined by qRT-PCR. **D**, PVT1 expression was significantly higher in osteosarcoma patients with metastasis than that without metastasis. **e**, Overall survival of patients with higher PVT1 was shorter than that with lower PVT1, p=0.0001, as measured by Kaplan-Meier analysis. **F**, Expression of PVT1 in 4 osteosarcoma cell lines, MG-63, U2OS, HOS, and 143B, was significantly higher than that in a normal human osteoblast cell line hFOB1.19. Data were shown as mean ± SD from three independent experiments. ***p<0.001 compared to controls, respectively.

significantly worse overall survival than those with low PVT1. Additionally, high PVT1 was shown to be closely correlated with advanced clinical stage (p = 0.008), tumor size (p = 0.009) and distant metastasis (p = 0.001) in patients with osteosarcoma (Table II).

Table II. Association of PVT1 expression with clinicopathological features of osteosarcoma.

Features	No. of cases	PVT1		*p*-value†
		High	Low	
Age at diagnosis				0.745
< 18	35	17	18	
≥ 18	13	7	6	
Gender				0.773
Female	27	12	11	
Male	21	12	13	
Histological subtype				0.849
Osteoblastic	14	8	7	
Chondroblastic	11	5	6	
Fibroblastic	12	7	5	
Mixed	11	5	6	
Clinical stage				0.008
I+IIA	19	5	14	
IIB/III	29	19	10	
Distant metastasis				0.001
Absent	21	5	16	
Present	27	19	8	
Tumor size (cm)				0.009
< 5	21	6	15	
≥ 5	27	18	9	
Anatomic location				0.505
Tibia/femur	36	17	19	
Elsewhere	12	7	5	

†*p*-value obtained from Pearson Chi-Square test.

PVT1 Promotes Migration and Invasion in HOS and 143B Cells

To functionally explore the role of PVT1 in osteosarcoma, we first transfected PVT1 siRNAs or a scramble control (siSCR) and PVT1-overexpressing plasmid (PVT1) or a vector control into HOS and 143B cells, respectively. As the outcomes of qRT-PCR assays presented in Figure 2a-d, PVT1 was successfully up- and down-regulated in HOS and 143B cells, individually. Further, we applied transwell assays with or without Matrigel to determine the migrative and invasive ability changes in osteosarcoma cells. As the representative photos displayed in Figure 2e-f, up- and down-regulation of PVT1 positively regulated cells migration and invasion in HOS and 143B cells.

MiR-486 is Downregulated and Involved in PVT1-Mediated Metastasis in HOS and 143B Cells

It is well known that lncRNAs function as miRNAs sponges to regulate their downstream genes. Therefore, we wondered whether any miRNAs were also involved in PVT1-mediated metastasis in osteosarcoma. We applied 3 online prediction software- Diana-Lncbase, MiRDB, and RegRNA, to screen miRNAs that might interact with PVT1. As presented in Figure 3a, we filtered out 6 miRNAs for further research. Through qRT-PCR detection and *in situ* hybridization (ISH) assay, we found that miR-486 was stably downregulated in 3 osteosarcoma tissue samples (Figure 3b-c). Meanwhile, through a further cellular level detection, miR-486 was displayed to be downregulated in 4 osteosarcoma cell lines, compared with that in a normal human osteoblast cell line hFOB 1.19 (Figure 3d). Even more, miR-486 was shown to be inversely correlated with PVT1 in osteosarcoma (Figure 3e). Functionally, upregulation of miR-486 (cotransfection of PVT1 and miR-486 mimics) was shown to reverse the facilitative effect of PVT1 on migration and invasion in HOS and 143B cells, and vice versa (Figure 3f-g).

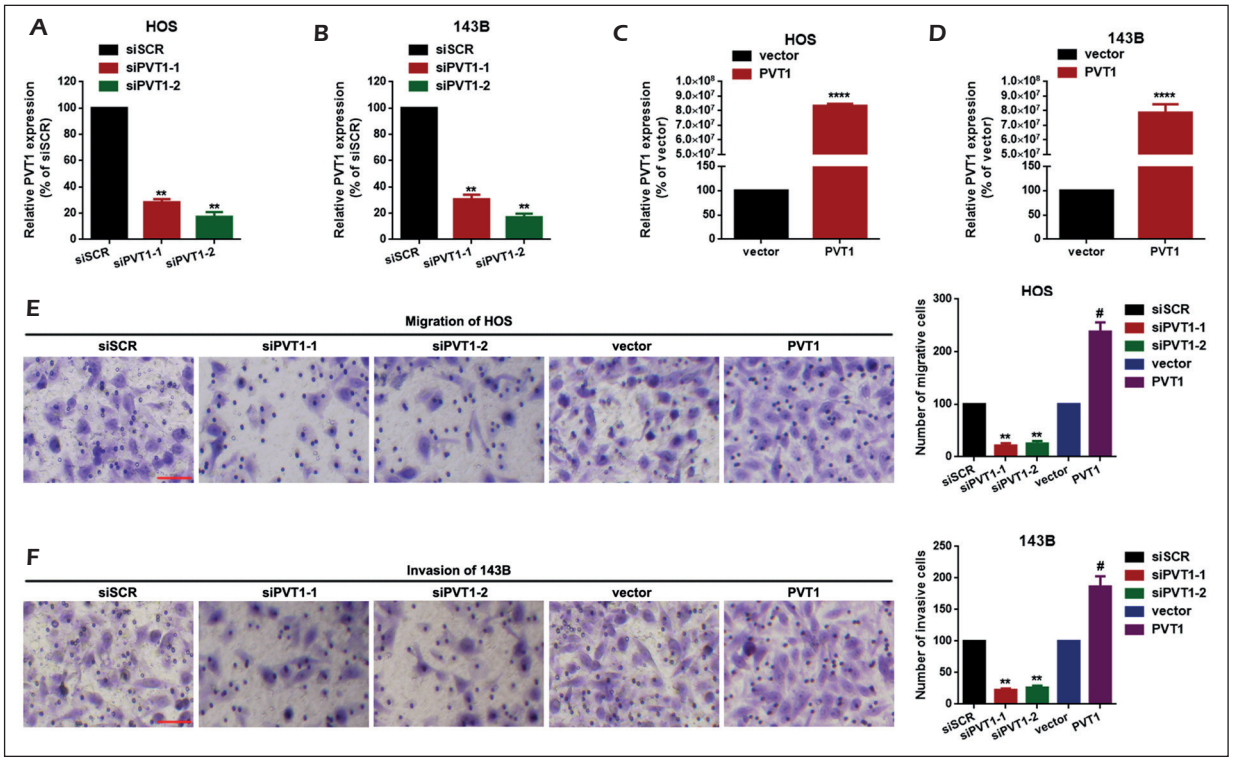

Figure 2. PVT1 promotes migration and invasion in HOS and 143B cells. **A-B**, PVT1 in HOS (**A**) and 143B (**B**) cells was knocked down by specific PVT1 siRNAs siPVT1-1 and siPVT1-2 as determined by qRT-PCR assay. **c-d**, PVT1 in HOS (**C**) and 143B (**D**) cells was increased by transfection of PVT1 overexpression plasmids, measured by qRT-PCR assay as well. **E-F**, Down- and up-regulation of PVT1 positively regulated osteosarcoma cells migration (**E**) and invasion (**F**) in HOS and 143B cells. The migrated or invaded cells were counted in 10 randomly chosen microscopic fields (magnification 200×) of each experiment and pooled. Data were shown as mean ± SD from three independent experiments. $^{**}p<0.01$ as normalizing and comparing with siSCR or vector group, individually.

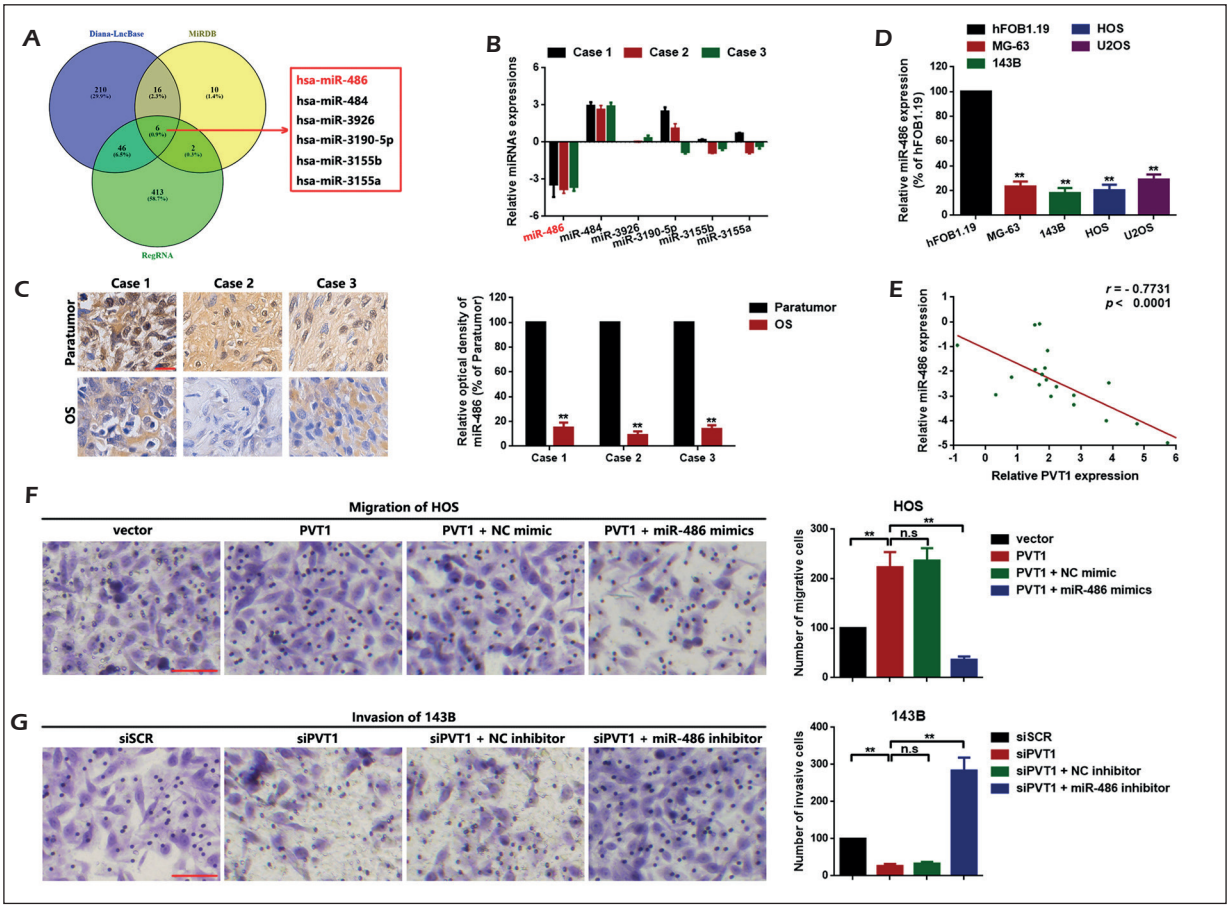

Figure 3. MiR-486 is downregulated and involved in PVT1-mediated metastasis in HOS and 143B cells. **A**, Schematic illustration exhibiting overlapping of the target miRNAs of PVT1 predicted by Diana-Lncbase, miRDB, and RegRNA. **B**, Relative levels of 6 miRNA candidates in the HOS and 143B lysates were determined by qRT-PCR. MiR-486 was stably downregulated in all 3 osteosarcoma tissue samples. **C**, *In situ* hybridizations (*IS*H) assay indicated that miR-486 was downregulated in osteosarcoma samples as compared with that in paratumor samples (magnifications 200×, scale bar 100 μm). **D**, Compared to hFOB1.19, miR-486 was downregulated in 4 osteosarcoma cell lines– G-63, U2OS, HOS, and 143B. **e**, Correlation analysis of PVT1 and miR-486 in osteosarcoma samples was conducted via Pearson's correlation coefficient. **F-G**, Transwell migration assay (**F**) and invasion assay (**G**) showed that the migration/invasion abilities of HOS and 143B cells were enhanced by overexpression of PVT1, while the facilitative effect of PVT1 was reversed by an upregulation of miR-486 (PVT1 + miR-486 mimics). The migrated or invaded cells were counted in 10 randomly chosen microscopic fields (magnification 100×) of each experiment and pooled. Each sample was run in triplicate and in multiple experiments for mean ± SD. $^{**}p<0.01$ compared to controls, respectively.

PVT1 is a Target of MiR-486 and Facilitates Metastasis Via MiR-486 Sponging

In this section, we tried to explore the relationship between PVT1 and miR-486. First, we demonstrated that PVT1 interacted with miR-486 in a reciprocal inhibition manner (Figure 4a-d). Second, we applied Luciferase assay to verify the targeted binding effect between miR-486 and PVT1 3' untranslated region (3'UTR). As shown in Figure 4e-g, the Luciferase activities were significantly weakened after co-transfection of wild reporter plasmid PVT1-wt (containing a miR-486 seeding region) and miR-486 mimics. While Luciferase activities were enhanced when the co-transfection of a mutant reporter plasmid PVT1-mut (containing a mutated miR-486 seeding region) and miR-486 mimics. These findings strongly verified that miR-486 could directly target to PVT1 3'UTR *via* the seeding region. Lastly, through transwell assay (Figure 4h-i), we illustrated that only wild type of PVT1-overexpressing plasmid (PVT1) promoted osteosarcoma cells migration and invasion. When the theoretical binding sites of miR-486 (miR-486 response elements, MREs-486) were mutated (transfection of PVT-mut), the facilitative effect was dismissed.

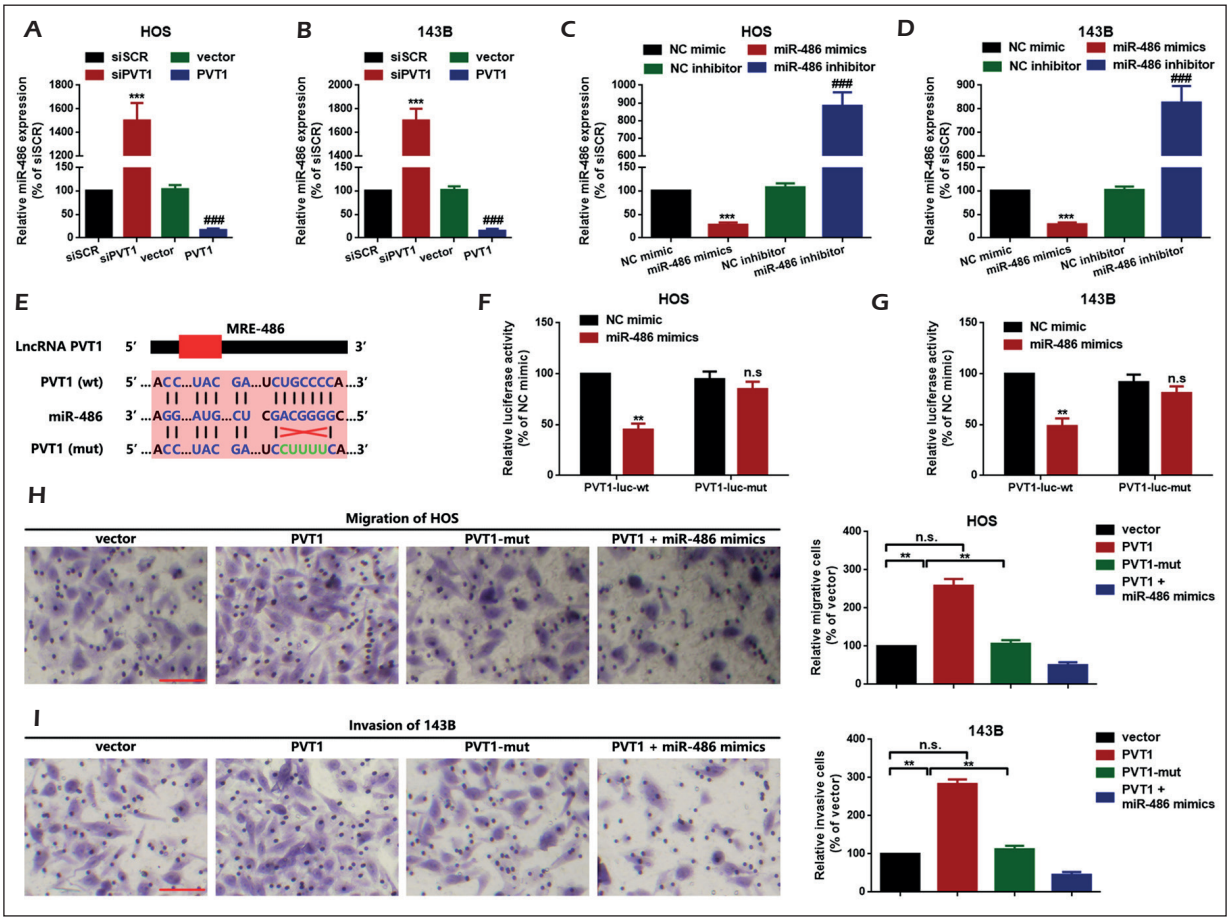

Figure 4. PVT1 is a target of miR-486 and facilitates metastasis via miR-486 sponging. **A-B**, Expression of miR-486 was inversely regulated by PVT1 as checked with qRT-PCR assay in HOS (**A**) and 143B (**B**) cells. **C-D**, Up- and down-regulation of miR-486 also negatively regulated PVT1 expression in HOS (**C**) and 143B (**D**) cells, measured by qRT-PCR assay. **e**, Sequence alignment of PVT1 reported plasmids (wt) containing wild and mutant miR-486 seed region. **F-G**, Targeted binding effect between miR-486 and PVT1 was confirmed via Luciferase assay. **H-I**, Transwell assay was performed to determine the changes of migration and invasion ability after different PVT1 or miR-486 intervention. The migrated or invaded cells were counted in 10 randomly chosen microscopic fields (magnification 100×) of each experiment and pooled. Each sample was run in triplicate and in multiple experiments for mean ± SD. $^{**}p<0.01$ compared to controls, respectively.

Furthermore, the promotive effect of PVT1 on osteosarcoma cells migration and invasion was found to be attenuated by an upregulation of miR-486 (cotransfection of PVT1-overexpressing plasmid and miR-486 mimics). In brief, all the results strongly indicated that PVT1 facilitated metastasis *via* miR-486 sponging in osteosarcoma cells.

Discussion

As a member of lncRNA family, PVT1 is encoded by the human PVT1 gene (also known as the PVT1 oncogene), and PVT1 was primarily reported as an activator of MYC in murine plasmacytoma variant translocations in 1984[24]. PVT1 exhibits the ability to increase proliferation and inhibit apoptosis during tumorigenesis in multiple cancers. Also, PVT1 serves as a novel predictor for metastasis in human malignancies[25,26]. An important reason for poor prognosis of osteosarcoma is the early stage of lung metastasis. Therefore, we focused on the relationship between PVT1 and metastasis of osteosarcoma. Through a series of qRT-PCR detection and statistical analysis, PVT1 was found to be upregulated in osteosarcoma tissue specimens and cell lines, and closely correlated with several poor clinicopathological features of osteosarcoma, like advanced clinical staging and distant metastasis. These data indicate that PVT1 might contribute to osteosarcoma progression. Recent

researches have shown that PVT1 also works as a metastasis-related oncogene in human cancers. Chen et al[27] reported that PVT1 was upregulated in gallbladder cancer (GBC) and promoted GBC proliferation and metastasis *via* regulating miR-143/HK2 axis. We also performed a loss of function transwell assay to evaluate the function of PVT1 working on osteosarcoma cells metastasis. We found that an upregulation and a knockdown of PVT1 positively regulated osteosarcoma cells migration and invasion, remarkably.

LncRNAs allow highly specific interactions that are capable of regulating various steps in the post-transcriptional process of mRNAs, including their splicing, editing, transport, translation, and degradation by recognizing complementary sequences of mRNAs[28]. The molecular mechanisms of action for PVT1 mainly include participating in DNA rearrangements, interacting with MYC, and encoding and co-working with miRNAs[29]. In recent years, a most popular theory for lncRNAs is ceRNA, firstly proposed by Salmena et al[30]. CeRNA theory presumes that messenger RNAs, transcribed pseudogenes, and lncRNAs "talk" to each other using microRNA response elements (MREs) as letters of a new language. Currently, we also hypothesize that PVT1 might co-work with any miRNAs in osteosarcoma. Through online prediction, we locked miR-486 for further study.

MiRNAs, 19-22 nucleotide in length, are a group of single-stranded and conserved non-coding RNAs. MiRNAs regulate their target genes at both post-transcriptional and translational levels by acting on the 3'-untranslated region (UTR). MiRNAs negatively regulate gene expression *via* decreasing the stability of target RNAs or limiting their translation[31]. MiR-486, also named as miR-486-1, is located at human chromosome 8p11.21 and contains one exon. Aberrant expression of miR-486 is closely associated with different types of human cancer, such as colorectal cancer, pancreatic cancer, hepatocellular carcinoma, lung cancer, and osteosarcoma[32-37]. In the current research, we measured the expression of miR-486 in osteosarcoma, and as previously reported, miR-486 was also demonstrated to be downregulated in osteosarcoma[38]. We further assessed the role of miR-486 on PVT1 mediated metastasis, and we found that an upregulation of miR-486 reversed PVT1-induced metastasis. Furthermore, we illustrated that miR-486 could target to PVT1 *via* theoretical MRE. Lastly, we found that the promotive effect of wild PVT1 overexpressing plasmid (PVT1) on osteosarcoma cells metastasis was attenuated by miR-486 mimics. These findings strongly suggested that PVT1 promoted osteosarcoma cells metastasis *via* miR-486 sponging.

Conclusions

Altogether, metastasis of osteosarcoma is an intricate biological process. Numerous molecules and signal pathways contribute to this complex process. Our findings indicate that PVT1/miR-486 axis might be a new molecular target for the treatment of osteosarcoma.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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