Downregulation of IncRNA PVT1 inhibits proliferation and migration of mesothelioma cells by targeting FOXM1

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Received August 13, 2021; Accepted October 12, 2021

DOI: 10.3892/or.2021.8238

Abstract. Malignant mesothelioma is a highly aggressive tumor, and an effective strategy for its treatment is not yet available. Long non-coding RNAs (lncRNAs) have been reported to be associated with various biological processes, including the regulation of gene expression of cancer-related pathways. Among various lncRNAs, plasmacytoma variant translocation 1 (PVT1) acts as a tumor promoter in several human cancers, but its mechanism of action has not yet been elucidated. Increased PVT1 expression was identified in ACC-MESO-1, ACC-MESO-4, CRL-5915, and CRL-5946 mesothelioma cell lines. PVT1 expression was investigated in mesothelioma cell lines by reverse transcription-quantitative polymerase chain reaction and its functional analysis by cell proliferation, cell cycle, cell migration, and cell invasion assays, as well as western blot analysis of downstream target genes. Knockdown of PVT1 expression in these cell lines by small interfering RNA transfection resulted in decreased cell proliferation and migration and increased the proportion of cells in the G2/M phase. The results of reverse transcription-quantitative polymerase chain reaction analysis revealed that PVT1 knockdown in mesothelioma cell lines caused the downregulation of Forkhead box M1 (FOXM1) expression, while the results of western blot analysis revealed that this knockdown reduced FOXM1 expression at the protein level. In addition, combined knockdown of PVT1 and FOXM1 decreased the proliferation of mesothelioma cell lines. In conclusion, PVT1 and FOXM1 were involved in the proliferation of cancer cells. Therefore, PVT1-FOXM1 pathways may be considered as candidate targets for the treatment of malignant mesothelioma.

Introduction

Malignant mesothelioma is a highly aggressive tumor with poor prognosis. It arises from mesothelial cells lining the serous cavities (pleura, pericardium, peritoneum and tunica vaginalis). The incidence of mesothelioma is increasing worldwide due to previous occupational and/or environmental exposure to asbestos (1,2). The incidence of malignant mesothelioma in Japan is predicted to reach a peak between 2030 and 2034. In developing countries, the incidence of this disease is predicted to increase due to the continued use of asbestos (3,4). Currently available treatments have a limited effect on malignant mesothelioma management (5). Therefore, there is a need to identify feasible and effective therapeutic targets.

Non-coding RNAs are RNA molecules that are transcribed from the genome but do not encode proteins. They have been revealed to play structural and functional roles within the cell (6-10). They are primarily grouped into two classes based on transcript size: Small non-coding RNAs and long non-coding RNAs (lncRNAs) (11). Small non-coding RNAs include microRNAs (miRNAs) that function as major regulators of gene expression and complex components of cellular gene expression networks. In contrast to miRNAs, lncRNAs are a class of RNA transcripts that are over 200 nucleotides in length (12). IncRNAs have been associated with various biological processes, including epigenetics, alternative splicing, and nuclear import; additionally, they function as precursors of small non-coding RNAs, and regulators of mRNA decay (13-15). Dysregulated IncRNA expression has been reported in numerous cancers, suggesting that IncRNAs are a newly emerging class of oncogenic and tumor-suppressor genes (16).

Plasmacytoma variant translocation 1 (PVT1) is an oncogenic IncRNA located at chromosomal region 8q24 (17). The carcinogenicity of PVT1 has been identified in various human cancers, including non-small cell lung (18), leukemia (19), hepatocellular (20), colon (21), breast (22), and ovarian cancer (23). Non-coding RNA expression data from Human Transcriptome 2.0 GeneChip Array analysis performed in our previous study revealed increased PVT1 expression in epithelioid mesothelioma and lung adenocarcinoma (24). In the present study, the biological function of PVT1 in mesothelioma was elucidated.
Materials and methods

**PVT1 expression database.** Affymetrix mRNA expression subset data were obtained from the Cancer Cell Line Encyclopedia (CCLE) website (data created from https://www.broadinstitute.org/ccle/ on December 7, 2019). The CCLE project dataset is a compilation of gene expression data from human cancer cell lines (25).

**Mesothelioma cell lines.** ACC-MESO-1 (Exasy ID: CV CL_5113) and ACC-MESO-4 (Exasy ID: CVCL_5114) mesothelioma cell lines were purchased from RIKEN BioResource Research Center (Tsukuba, Japan), and NCI-H2052 (CRL-5915) and NCI-H2452 (CRL-5946) mesothelioma cell lines were purchased from the American Type Culture Collection (ATCC). In addition, two lung adenocarcinoma cell lines, A549 and PC9, purchased from the European Collection of Authenticated Cell Cultures, were also used to confirm PVT1 expression in lung adenocarcinoma. Cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium supplemented with 1% kanamycin, 1% amphotericin B, and 10% fetal bovine serum (FBS; all from Thermo Fisher Scientific, Inc.). Cells were maintained in culture dishes at 37°C in a humidified incubator supplemented with 5% CO2.

**Transfection of mesothelioma cells.** PVT1 small interfering (si)RNA (Lincode Human PVT1 siRNA - SMARTpool; cat. no. R-029357-00-0005) and its negative control (NC) siRNA (Lincode Non-targeting Pool; cat. no. D-001320-10-05) were purchased from GE Healthcare Dharmacon, Inc. Forkhead box M1 (FOXM1) siRNA (FOXM1 Silencer Select Pre-designed siRNA; cat. no. 4427037 ID# s5248) and its NC siRNA (Silencer Select Negative Control No. siRNA; cat. no. 4390843) were purchased from Thermo Fisher Scientific, Inc. Cells cultured until attaining 70-80% confluency, were transfected with 50 nM of PVT1/NC siRNA, 25 nM of FOXM1/NC siRNA, or both, using Lipofectamine RNAiMAX (Thermo Fisher Scientific, Inc.) in Opti-Mem Reduced Serum Medium (Thermo Fisher Scientific, Inc.) at 37°C in a humidified incubator supplemented with 5% CO2, according to the manufacturer's recommended protocols. The images of morphological change of the transfected mesothelioma cells were captured at 0 and 72 h using a CKX53 inverted light microscope with a DP21 digital camera (Olympus Corporation).

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Mesothelioma cell lines (3x10^5 cells) were transfected with 15 pmol of PVT1/NC siRNA or 7.5 pmol of FOXM1/NC siRNA in 6-well plates at 37°C in a humidified incubator supplemented with 5% CO2 for 72 h. RNA was extracted from the cells using Maxwell® RSC simplyRNA Cells Kit and Maxwell® RSC Instrument (both from Promega Corporation) according to the manufacturer's protocols. The extracted RNA was reverse-transcribed with SuperScript IV VILO Master Mix (Thermo Fisher Scientific, Inc.) and amplified using Power Up SYBR Green Master Mix (Thermo Fisher Scientific, Inc.) on an AriaMx Real-Time PCR System (Agilent Technologies, Inc.) according to the manufacturer's recommended protocols. In brief, qPCR was performed with initial denaturation at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 sec and annealing and elongation at 60°C for 1 min, and a dissociation curve condition from 95°C to 60°C. Relative expression levels were calculated using the comparative 2^-ΔΔCT method (26). Expression levels were normalized against those of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The primer sequences used for RT-qPCR were as follows:

- **PVT1 forward**, 5'-TGAGAACTGTCCTTAAGCTGACC-3' and reverse, 5'-AGAGCAGAGCTGGGTTC-3';
- **FOXM1 forward**, 5'-GGAGCAGCGAGATTTGTAAGG-3' and reverse, 5'-GTGTATGCGAATATTGTATCAGG-3'; and
- **GAPDH forward**, 5'-ACAACTTTGGAATCATGTAGG-3' and reverse, 5'-GCCATACGCACAGTTTGGC-3'.

**Cell proliferation assay.** Mesothelioma cell lines (3x10^4 cells) were incubated with 1 pmol PVT1/NC siRNA or 0.5 pmol FOXM1/NC siRNA in 96-well plates at 37°C in a humidified incubator supplemented with 5% CO2 for 3 days. The proliferation rate was determined at 24, 48 and 72 h using 100 µl of 2X Cell Titer-Glo 2.0 reagent (Promega Corporation), which assesses the number of viable cells relative to the ATP level, with a GloMax Explorer microplate reader (Promega Corporation) according to the manufacturer's protocols.

**Cell cycle assay.** Mesothelioma cell lines (1x10^5 cells) were transfected with 5 pmol PVT1/NC siRNA in 24-well plates for 3 days, and subsequently the cells were collected after trypsinization and fixed in 70% ethanol in 15-ml centrifuge tubes at room temperature for ~3 h. After ethanol removal, the cells were stained with 200 ml of Guava Cell Cycle Reagent (Luminex Corporation) at room temperature shielding away from the light for 30 min. The reagent containing propidium iodide discriminates the cells at different stages of the cell cycle by labeling cellular DNA. The labeling signal intensity was evaluated using a Guava EasyCyte Mini flow cytometer (Guava Technologies according to the manufacturer's protocols. Analysis of raw data was performed with FCS express 5.0 (De Novo Software).

**Wound healing assay.** The migration ability of all four mesothelioma cells was analyzed using a wound scratch assay. Serum starved mesothelioma cell lines grown to 80% confluence were incubated overnight with 5 pmol PVT1/NC siRNA in collagen-coated 24-well plates at 37°C in a humidified incubator supplemented with 5% CO2. Wounds were created by scratching the cells with 1-ml micropipette tips. The wells were washed twice to remove floating cells. Images of the gap area (wound) were captured every 24 h (for ACC-MESO-1 every 12 h) using a CKX53 inverted light microscope equipped with a DP21 digital camera (Olympus Corporation), and the gap area was further analyzed using T Scratch software version 1.0 downloaded from https://github.com/cselab/TScratch (27).

**Cell invasion assay.** BD FluroBlok culture inserts containing 8-μm pores (BD Biosciences) were coated with 100 µl of 10X diluted Geltrex Matrigel (Thermo Fisher Scientific, Inc.) at 37°C in a humidified incubator supplemented with 5% CO2 for 3 h. Mesothelioma cell lines (3x10^4 ACC-MESO-1 cells, and 5x10^4 ACC-MESO-4, CRL-5915, CRL-5946 cells) were incubated with 3 pmol siRNA in 500 µl RPMI-1640 medium (without FBS) in the upper chamber of culture inserts and 750 µl RPMI-1640
medium containing 5% FBS in the lower chamber of culture inserts according to the manufacturer’s protocols. Cells were incubated at 37°C in a humidified incubator supplied with 5% CO₂ for 72 h (48 h for ACC-MESO-1 cells), and invading cells were stained with addition of 50 µl of 1 µg/ml solution of Hoechst 33324 (Thermo Fisher Scientific, Inc.) at room temperature for 10 min, and subsequently the imaged area of the insert membrane was visualized using a fluorescence microscope. The total number of invading cells was analyzed using the CellProfiler cell imaging software version 2.1.0 downloaded from https://cellprofiler.org (28).

Western blot analysis. Mesothelioma cell lines (3x10⁵ cells) were transfected with 15 pmol PVT1/NC siRNA in 6-well plates for 72 h. Cell lysates were obtained from the cells using RIPA Lysis Buffer System (Santa Cruz Biotechnology, Inc.), and total protein was determined with Qubit™ Protein Assay Kit using a Qubit Fluorometer (Thermo Fisher Scientific, Inc.). Total proteins (20 µg) were electrophoresed on a 10% sodium dodecyl sulfate-polyacrylamide gel (SureCast Acrylamide Gel; Thermo Fisher Scientific, Inc.) at 200 V for 40 min and transferred onto polyvinylidene difluoride (PVDF) membranes using a Mini Blot Module (Thermo Fisher Scientific, Inc.) at 20 V for 60 min. Following blocking with 2% bovine serum albumin (Sigma Aldrich; Merck KGaA) in 1X TBS with 0.05% Tween-20 at room temperature for 1 h, the membranes were incubated overnight at 4°C with primary antibodies [anti-FOXM1 rabbit monoclonal antibody (1:4,000; product no. 20459S) and an anti-GAPDH rabbit monoclonal antibody (1:4,000; product no. 2118S; both from Cell Signaling Technology, Inc.)]. The membranes were then incubated with the anti-rabbit IgG, HRP-linked secondary antibody (1:4,000; cat. no. 7074P2; Cell Signaling Technology, Inc.) at room temperature for 40 min. The membranes were stained with ImmunoStar LD (Wako Pure Chemical Industries) at room temperature for 1 min and images were captured using a c-Digit Blot Scanner (LI-COR). Scanned images were analyzed by Image Studio Digits software version 5.2 (LI-COR Biosciences).

Statistical analysis. The experiments were performed at least three times in triplicate. Experimental data are expressed as the mean ± standard deviation. The statistical significance of the difference between two groups was analyzed using unpaired Student's t-test with the default function of Microsoft Excel version 16.53. P<0.05 was considered to indicate a statistically significant difference.

Results

PVT1 expression in mesothelioma and lung adenocarcinoma cell lines. PVT1 expression was high in mesothelioma and non-small cell cancers, in addition to different human cancers (Fig. 1A). RT-qPCR analysis results revealed that PVT1 was expressed in all four mesothelioma cell lines and two
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lung adenocarcinoma cell lines. Compared with the average PVT1 expression in the two lung adenocarcinoma cell lines, PVT1 expression was increased by 1.8-, 1.8-, 2.4-, and 1.8-fold in ACC-MESO-1, ACC-MESO-4, CRL-5915 and CRL-5946 cell lines, respectively (Fig. 1B).

PVT1 expression is reduced by siRNA transfection. PVT1 expression was downregulated by >80% following PVT1 siRNA transfection in all mesothelioma cell lines compared with that in cells transfected with NC siRNA (Fig. 2A). Morphological changes were not observed in PVT1 siRNA-transfected mesothelioma cell lines compared with NC siRNA-transfected mesothelioma cell lines (Fig. 2B).

PVT1 knockdown reduces mesothelioma cell proliferation and increases the G2/M phase of the cell cycle. Knockdown of PVT1 significantly reduced the proliferation of all mesothelioma cells compared with NC siRNA-transfected cells.
Following 3 days of treatment, the inhibition of PVT1 expression significantly reduced the viability of ACC-MESO-1 cells by 22.9%, ACC-MESO-4 cells by 14.8%, CRL-5915 cells by 17.6%, and CRL-5946 cells by 23.3% (Fig. 3A). The proportion of cells in the G2/M phase in the PVT1 siRNA-transfected mesothelioma cell lines (28.7, 27.4, 21.0, and 30.3% in ACC-MESO-1, ACC-MESO-4, CRL-5915, and CRL-5946 cell lines, respectively) was significantly higher than with the NC siRNA-transfected mesothelioma cell lines (17.5, 15.6, 14.4, and 22.8%). The proportion of cells in the G1 phase in the PVT1 siRNA-transfected mesothelioma cell lines (28.7, 27.4, 21.0, and 30.3% in ACC-MESO-1, ACC-MESO-4, CRL-5915, and CRL-5946 cell lines, respectively) was significantly higher than with the NC siRNA-transfected mesothelioma cell lines (17.5, 15.6, 14.4, and 22.8%).
PVT1 knockdown reduces mesothelioma cell migration but not invasion. In the PVT1 siRNA-transfected cells, the gap area decreased more slowly than in the NC siRNA-transfected cell lines in all four cell lines. The migration of ACC-MESO-1 cells after 24 h of PVT1 knockdown was reduced by 67.5% and that of ACC-MESO-4, CRL5915, and CRL5946 cell lines after 48 h of PVT1 knockdown was reduced by 64.2, 26.8 and 27.3%, respectively (Fig. 4).

PVT1 knockdown downregulates FOXM1 expression. All four mesothelioma cell lines exhibited FOXM1 expression. FOXM1 mRNA expression in cells transfected with PVT1 siRNA compared with cells transfected with NC siRNA was downregulated by 77, 83, 84 and 82% in ACC-MESO1, ACC-MESO4, CRL-5915, and CRL-5946 cell lines, respectively (Fig. 5A). Similarly, FOXM1 protein was downregulated by 41% in ACC-MESO-1 cells, 35% in ACC-MESO-4 cells, 56% in CRL-5915 cells, and 55% in CRL-5946 cells (Fig. 5B).

FOXM1 and PVT1 knockdown reduces mesothelioma cell proliferation. Transfection with either FOXM1 or PVT1 siRNA revealed a similar decrease in the proliferation of mesothelioma cells. However, combined FOXM1 and PVT1 siRNA transfection further decreased the proliferation of mesothelioma cells. Following 3 days of treatment, FOXM1 knockdown significantly
Figure 5. (A) FOXM1 expression of PVT1 or NC siRNA transfection as determined by reverse transcription‑quantitative PCR. The lower panel reveals amplification curves of PVT1 expression with dissociation curves. (B) FOXM1 expression as determined by western blot analysis in PVT1 or NC siRNA‑transfected cells. FOXM1, Forkhead box M1; PVT1, plasmacytoma variant translocation 1; siRNA, small interfering RNA; NC, negative control.

Figure 6. Cell proliferation assay of mesothelioma cell lines transfected with PVT1 siRNA, FOXM1 siRNA and combination of both siRNAs for three days. FOXM1, Forkhead box M1; PVT1, plasmacytoma variant translocation 1; siRNA, small interfering RNA; NC, negative control.
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reduced the viability of ACC-MESO-1, ACC-MESO-4, CRL-5915, and CRL-5946 cells by 24.8, 19.0, 19.2 and 24.4%, respectively. Furthermore, inhibition of both PVT1 and FOXM1 expression significantly reduced the viability of ACC-MESO-1, ACC-MESO-4, CRL-5915, and CRL-5946 cells by 37.9, 35.7, 29.1 and 39.9%, respectively (Fig. 6).

PVT1 and FOXM1 knockdown downregulates FOXM1 expression. Downregulation of FOXM1 mRNA expression in cells transfected with combined PVT1 and FOXM1 siRNA compared with cells transfected with NC siRNA (86, 88, 80 and 82% in ACC-MESO-1, ACC-MESO-4, CRL-5915, and CRL-5946 cell lines, respectively) was markedly lower than that in cells transfected with PVT1 siRNA alone (64, 73, 62 and 65%) but similar to that in cells transfected with FOXM1 siRNA alone (84, 86, 79 and 81%) (Fig. 7A). Downregulation of FOXM1 protein expression in cells transfected with combined PVT1 and FOXM1 siRNA compared with cells transfected with NC siRNA (77, 72, 74 and 75% in ACC-MESO-1, ACC-MESO-4, CRL-5915, and CRL-5946 cell lines, respectively) was lower than that in cells transfected with PVT1 siRNA alone (39, 30, 54 and 46%) but similar to that in cells transfected with FOXM1 siRNA alone (72, 65, 70 and 73%) (Fig. 7B).

Discussion

Malignant pleural mesothelioma (MPM) is an aggressive form of cancer. Patients with malignant mesothelioma are treated with surgery, radiotherapy, chemotherapy, and targeted drug therapy. However, the survival rates of MPM patients remain extremely low, with survival ranging from 5 to 13.2 months (29).

In a previous study, the median survival period did not improve beyond 13-29 months with extended pleurectomy/decortication and 12-22 months with extrapleural pneumonectomy (30). Therefore, feasible and effective therapeutic targets need to be identified. In the present study, the biological function of PVT1-FOXM1 was investigated as a possible novel target in malignant mesothelioma.

As they regulate gene expression and function at the transcriptional, translational, and post-translational levels, lncRNAs are important in tumor growth and metastasis (31,32). Wright et al have previously revealed various dysregulated lncRNAs involved in the pathogenesis of malignant mesothelioma using NCode long noncoding microarrays and their potential to serve as biomarkers in MPM (33). However, the mechanisms of these lncRNAs have not yet been described in detail. Non-coding transcripts from our previous gene expression microarray analysis of malignant mesothelioma and lung adenocarcinoma were extracted and analyzed and numerous upregulated lncRNAs were identified, including PVT1, MEG3, and H19 (24). Riquelme et al previously suggested that c-Myc and PVT1 copy number gain may promote a malignant phenotype of mesothelioma with PVT1, demonstrating a tendency to upregulate proliferation and inhibit apoptosis (34). The biological functions of PVT1 in malignant mesothelioma have not been fully established; however, previous studies have revealed that PVT1 knockdown inhibits cell proliferation and induces apoptosis through suppression of c-Myc in leukemia (19) and breast cancer (22). PVT1 binds competitively with microRNA-424, which has been reported to increase radiosensitivity by regulating CARM1 in non-small cell lung cancer (18). PVT1 led to increased proliferation and invasion of glioma (35) and hepatocellular carcinoma (20) by targeting EZH2. In the present study, increased expression of PVT1 in mesothelioma and lung adenocarcinoma cell lines was revealed by RT-qPCR, and PVT expression was revealed to be ~2 times higher in mesothelioma than in lung...
adenocarcinoma cell lines. PVT1 knockdown of mesothelioma cell lines revealed reduced cell proliferation with G2/M arrest and migration.

FOXMI, a member of the FOX transcription factor family 1, is associated to cell viability and is considered a key gene in the carcinogenic pathway. Previous studies have indicated that FOXMI participates in drug resistance, cancer, and metastasis of cancers (36-38). Several previous studies have demonstrated that FOXMI is overexpressed in multiple cancers, such as ovarian (39), colon (40), gastrointestinal (41), and non-small cell lung cancer (42). Increased FOXMI expression was also observed in mesothelioma cell lines, and knockdown of mesothelioma cell lines decreased their proliferation.

PVT1 was revealed to promote tumor progression by interacting with FOXMI in ovarian and gastric cancer (43,44). In the present study, it was also revealed that PVT1 knockdown in mesothelioma cell lines downregulated FOXMI expression.

Our study also revealed that PVT1 knockdown reduced FOXMI expression. Furthermore, knockdown of both FOXMI and PVT1 in mesothelioma cell lines demonstrated more reduced proliferation of mesothelioma cell lines compared with knockdown of PVT1 or FOXMI alone. FOXMI expression in mesothelioma cell lines with combined PVT1 and FOXMI knockdown was lower than that with PVT1 knockdown alone. Further studies such as spheroid formations and in-vivo experiments which are limited in this study are necessary to clarify the function of PVT1-FOXMI in mesothelioma cell lines.

In conclusion, it was revealed in the present study that lncRNA PVT1 was upregulated in mesothelioma cell lines, and knockdown of PVT1 decreased the proliferation and migration of mesothelioma cells and downregulated FOXMI expression. Furthermore, concurrent knockdown of FOXMI and PVT1 in mesothelioma cell lines demonstrated more reduced proliferation compared with knockdown of PVT1 or FOXMI alone. PVT1 and FOXMI may be considered as candidate targets for the therapy of malignant mesothelioma.

Acknowledgements

The authors would like to thank Ms Yukari Go and Mr Tatsuya Nakagawa of the Technical Center, Hiroshima University for their excellent technical assistance and Ms Naomi Fukuhara (Department of Pathology, Hiroshima University) for administrative support.

Funding

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

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

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