Inhibition of ZNF746 suppresses invasion and epithelial to mesenchymal transition in H460 non-small cell lung cancer cells

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Abstract. Although ZNF746, also known as Parkin-interacting substrate (PARIS), has been reported to suppress peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) and its target gene NRF-1 leading to the neurodegeneration in Parkinson's disease, its function in tumorigenesis has yet to be investigated. Thus, in the present study, the role of ZNF746 in the invasion and epithelial to mesenchymal transition (EMT) in H460 non-small cell lung cancer (NSCLC) cells was investigated. Invasion assay showed that inhibition of ZNF746 using siRNA transfection inhibited the invasion of H460 NSCLC cells using Boyden chamber. Quantitative PCR (qPCR) analysis revealed that the silencing of ZNF746 attenuated the expression of matrix metalloproteinase (MMP)1, MMP2 and MMP9, but not MMP7, in H460 NSCLC cells. Immunoblotting assay revealed that the expression of E-cadherin and β-catenin of epithelial phenotype was upregulated, while Slug was downregulated in ZNF746 siRNA-transfected H460 NSCLC cells. Accordingly, the mRNA expression of E-cadherin was upregulated while vimentin or Slug, Twist, ZEB as EMT key transcriptional factors were suppressed in ZNF746 siRNA-transfected H460 NSCLC cells. Also, mRNA expression of transcriptional marker Nanog and Octamer-binding transcription factor 4 (OCT4), known to enhance malignancy and metastasis in lung adenocarcinoma, was suppressed in ZNF746 siRNA-transfected H460 NSCLC cells. Notably, the endogenous expression of ZNF746 was induced in parallel with Twist at the protein level during hypoxia. Overall, our findings suggest that inhibition of ZNF746 suppresses the invasion and EMT molecules in H460 NSCLC cells and ZNF746 may be an important target molecule in lung tumorigenesis.

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Introduction

Lung cancer is one of the major causes of cancer-related mortality worldwide (1). Lung cancer is generally classified into two major types, small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). The latter is the most common type accounting for ~80% of lung cancer cases (2). The early diagnosis of lung metastasis can contribute to the decrease of mortality and morbidity, since the migration and invasion of cancer cells from the primary tumor sites into the surrounding tissues promote the progression of metastasis.

Of note, epithelial to mesenchymal transition (EMT) was known to promote the tumor cell infiltration into interstitial stroma by extending microtubule-based protrusions and inhibiting cell proliferation (3). There is accumulating evidence that EMT plays an important role in cancer progression, wound healing, invasion and tissue fibrosis as well as in embryogenesis (4-6). The EMT process results in the loss of epithelial cell phenotype and exhibits mesenchymal cell characteristics. Therefore, epithelial cell type genes such as E-cadherin, claudins and occludin are downregulated, while mesenchymal cell type genes including N-cadherin, vimentin and fibronectin related to the migration, invasion and proliferation are upregulated during the EMT process (7,8). However, although EMT transcriptional factors such as Twist, Snail, Slug and Zinc finger E-box binding homeobox 1 (ZEB1) are aberrantly overexpressed in a patient with NSCLC as a highly fibrotic malignancy (9,10), their upstream factors remain unclear.

ZNF746, which contains a Kruppel-associated box at its N terminus and a C2HC/C2H2 type zinc finger at its C terminus, interacts with Parkin as a ubiquitin E3 ligase (11,12) or as a suppressor of peroxisome proliferator-activated receptor γ coactivator-1 (PGC-1) in neurodegeneration in Parkinson's disease (12). Nevertheless, the role of ZNF746 has yet to be elucidated in tumorigenesis. Thus, in the present study, we investigated for the first time whether or not the inhibition of ZNF746 suppresses the invasion and EMT process in H460 NSCLC cells.

Materials and methods

Cell culture. H460 NSCLC cells (HTB-177™) were obtained from the American Type Culture Collection (ATCC). H460 NSCLC cells were maintained in Roswell Park Memorial

Institute (RPMI)-1640 media (WelGENE, Inc., Daegu, Korea) supplemented with 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA) and 1% antibiotics at 37˚C in 5% CO_2.

Invasion assay. To investigate the migratory properties of H460 NSCLC cells, after transfecting with control or small interfering RNA (siRNA) ZNF746 (Bioneer, Daejun, Korea) (80 nM) using INTERFERin® reagent (Polyplus, Illkirch, France), invasion assay was carried out using modified 48-well microchemotaxis chambers (Neuro Probe Inc., Gaithersburg, MD, USA). Polyvinylpyrrolidone-free polycarbonate filters (8 mm pore size) (Neuro Probe, Inc.) were coated with Matrigel, a solubilized basement membrane matrix. The lower chamber was filled with media containing 10% FBS as chemoattractant agents. The coated filter and upper chamber were laid over the lower chamber. The control siRNA and ZNF746 siRNA (Bioneer, Daejun, Korea)-transfected H460 NSCLC cells ($1x10^4$ cells/25 μl) were seeded onto the upper chamber wells. After 24 h incubation at 37˚C, the filter was fixed and stained with Diff-Quick (Sysmex, Kobe, Japan) and non-migrated cells on the upper surface of the filter were wiped off with a swab. Then, randomly chosen fields were photographed under a microscope (DFC420C; Leica, Germany) and the number of cells that migrated to the lower surface was counted.

Real-time quantitative RT-PCR (RT-qPCR). Total RNA was isolated from H460 NSCLC cells with QIAzol (Invitrogen, Carlsbad, CA, USA). A reverse transcription kit (Promega, Madison, WI, USA) was used to construct the template cDNA. RT-qPCR was performed with the LightCycler™ instrument (Roche Applied Sciences, Indianapolis, IN, USA) according to the manufacturer's protocol. The mRNA level of GAPDH was used to normalize the expression of genes of interest. The primers used were: E-cadherin forward, 5'-CAAG CTATCCTTGCACCTCAG-3' and reverse, 5'-GCATCAAGA GAACTCCTATCTTG-3'; matrix metalloproteinase (MMP)1 forward, 5'-CTGGCCACAACTGCCAAATG-3' and reverse, 5'-CTGTCCCTGAACAGCCCAGTACTTA-3'; MMP2 forward, 5'-TCTCCTGACATTGACCTTGGC-3' and reverse, 5'-CAAGGTGCTGGCTGAGTAGATC-3'; MMP7 forward, 5'-TGAGCTACAGTGGGAACAGG-3' and reverse, 5'-TCAT CGAAGTGAGCATCTCC-3'; MMP9 forward, 5'-TTGACA GCGACAAGAAGTGG-3' and reverse, 5'-GCCATTCACGT CGTCCTTAT-3'; Slug forward, 5'-GCGATGCCCAGTCTA GAAAA-3' and reverse, 5'-GCAGTGAGGGCAAGAAA AAG-3'; Nanog forward, 5'-CAGCTGTGTGTACTCAATG ATAGATTT-3' and reverse, 5'-ACACCATTGCATTTCTTCG GCCAGTTG-3'; octamer-binding transcription factor 4 (OCT4) forward, 5'-GACAACAATGAAAATCTTCAGG AG-3' and reverse, 5'-CTGGCGCCGGTTACAGAACCA-3'; SMAD2 forward, 5'-GTTCCTGCCTTTGCTGAGAC-3' and reverse, 5'-TCTCTTTGCCAGGAATGCTT-3'; vimentin forward, 5'-CAACCTGGCCGAGGACAT-3' and reverse, 5'-ACGCATTGTCAACATCCTGTCT-3'; ZNF746 forward, 5'-GCTGCACACCGGCGAGCGGCCC-3' and reverse, 5'-CT TGCGGAGGTGGTCCTTGCG-3'; GAPDH forward, 5'-CCA CTCCTCCACCTTTGAC-3' and reverse, 5'-ACCCTGTTGC TGTAGCCA-3'.

Immunofluorescence assay. H460 NSCLC cells transfected with control or ZNF746 siRNA (Bioneer) plasmids were fixed with 4% paraformaldehyde and permeabilized in 0.1% Triton X-100. The fixed H460 NSCLC cells were then washed with 1X PBS and blocked with 10% normal goat serum blocking solution (Zymed Laboratories, Carlsbad, CA, USA) for 30 min at room temperature. Fixed cells were incubated with the specific primary antibodies against ZNF746 (Sigma-Aldrich, St. Louis, MO, USA), E-cadherin, β-catenin (Cell Signaling Technology, Inc., Danvers, MA, USA) overnight at 4˚C. After washing, the cells were incubated with Alexa Fluor 594 goat anti-rabbit IgG (Invitrogen) for 30 min at room temperature. After washing, the cells were mounted with Vectashield/DAPI (Vector Laboratories, Inc., Burlingame, CA, USA) and visualized by a Carl Zeiss LSM5 confocal microscope.

Western blotting. Whole cell lysates from H460 NSCLC cells transfected with control or ZNF746 siRNA plasmids were prepared using lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1 mM EDTA, 1 mM Na_3VO_4, 1 mM NaF and protease inhibitor cocktail). The concentration of protein was measured by using a Bio-Rad DC protein assay kit II (Bio-Rad, Hercules, CA, USA). The proteins were separated on 8 or 12% Bis-Tris Gels (Invitrogen). Hybond ECL transfer membrane (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) was used to transfer the gel. The membranes were blocked with 5% nonfat dry milk. Primary antibodies were used for detecting ZNF746 (Sigma-Aldrich), E-cadherin, β-catenin (Cell Signaling Technology, Inc.), Twist (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), and β-actin (Sigma-Aldrich). Secondary antibody was used with horseradish peroxidase (HRP)-conjugated secondary antibodies. Enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech, Inc., Piscataway, NJ, USA) was used.

Results

Silencing of ZNF746 suppresses the invasive property in H460 NSCLC cells. To investigate the role of ZNF746 in tumorigenesis, the effect of the silencing of ZNF746 was evaluated on the invasion of H460 NSCLC cells transfected with ZNF746 siRNA plasmid. Boyden chamber invasion assay was performed to examine the effect on the invasive activity of ZNF746. Knockdown of ZNF746 significantly inhibited the invasive property compared to untreated control in H460 NSCLC cells (Fig. 1A and B). Western blot assay showed that ZNF746 was efficiently inhibited by siRNA transfection (Fig. 1C).

Silencing of ZNF746 attenuates the expression of MMP1, MMP2 and MMP9, but not MMP7, in H460 NSCLC cells by RT-qPCR. MMPs promote tumor survival, invasion as well as metastasis (13). In this regard, we evaluated whether ZNF746 depletion regulates the expression of MMPs (MMP1, MMP2, MMP7 and MMP9) in H460 NSCLC cells by RT-qPCR. The depletion of ZNF746 attenuated the expression of MMP1, MMP2 and MMP9, but not MMP7 (Fig. 2).

Silencing of ZNF746 upregulates E-cadherin and β-catenin in H460 NSCLC cells by western blotting and immunohistochemistry. EMT is a key phenotype by which cancer cells acquire

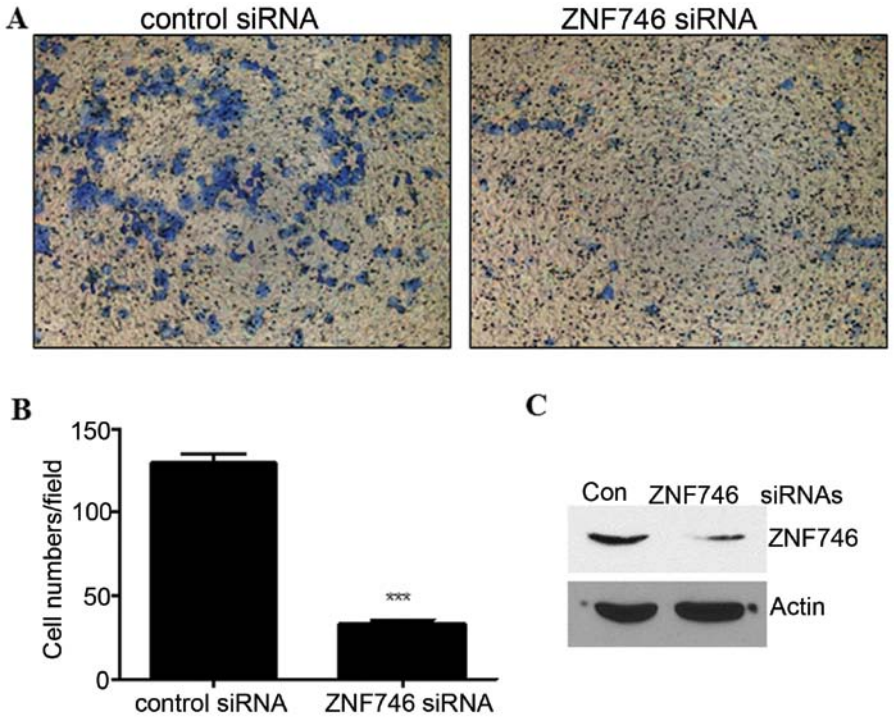

Figure 1. Silencing of ZNF746 inhibits the invasive property of H460 non-small cell lung cancer (NSCLC) cells. (A) The effect of ZNF746 siRNA transfection on the migrated cells of H460 NSCLC cells. (B) Bar graph for the migrated cells of H460 NSCLC cells transfected with control and ZNF746 siRNA plasmids. Data represent the means ± SD (N=3). ***P<0.001 vs. siRNA control. (C) Protein expression of ZNF746 and β-actin in control or ZNF746 siRNA-transfected H460 NSCLC cells by western blotting.

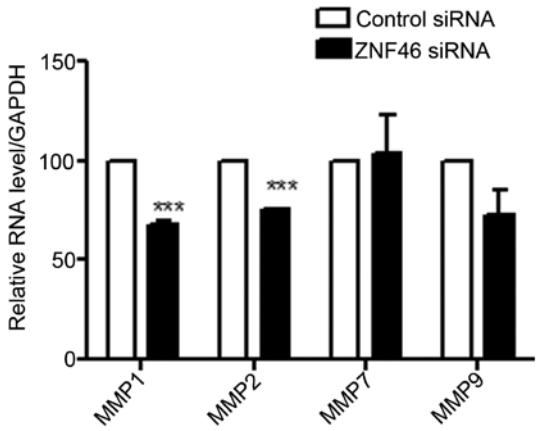

Figure 2. Silencing of ZNF746 attenuates the expression of matrix metalloproteinase (MMP)1, MMP2 and MMP9 but not MMP7 in H460 non-small cell lung cancer (NSCLC) cells. The expressions of MMP1, MMP2, MMP7 and MMP9 at the mRNA level were measured by real-time quantitative RT-PCR (RT-qPCR) and GAPDH was used for normalization. Data represent means ± SD (N=3). ***P<0.001 vs. siRNA control.

motility and invasion (4,8). In this regard, to explore whether or not ZNF746 is associated with EMT in H460 NSCLC cells, we examined the biomarkers of epithelial and mesenchymal phenotypes after ZNF746 siRNA knockdown in H460 NSCLC cells by western blotting. ZNF746 knockdown enhanced the expression of E-cadherin and β-catenin as epithelial markers, while the expression of Slug was suppressed as a mesenchymal marker (Fig. 3A). Consistently, immunohistochemistry revealed that the silencing of ZNF746 reduced the red color expression for β-catenin and E-cadherin in H460 NSCLC cells (Fig. 3B).

Silencing of ZNF746 upregulates EMT molecules at the mRNA level in H460 NSCLC cells and hypoxia enhances the protein expression of ZNF746 in parallel with Twist. To verify that ZNF746 regulates EMT molecules at the mRNA level, RT-qPCR analysis was performed. Expression of epithelial marker (E-cadherin), mesenchymal marker (vimentin) or transcriptional factors such as ZEB1, Twist, Snail and Slug, was evaluated in ZNF746 siRNA-transfected H460 NSCLC cells. ZNF746 knockdown downregulated the expression of Twist, vimentin, ZEB1, and Slug, but not SMAD2, and Snail, and also upregulated the expression of E-cadherin in H460 NSCLC cells (Fig. 4A and B).

Hypoxia accelerates the cell invasion (14) and plays an important role in EMT (15). To confirm that ZNF746 or Twist can be induced under hypoxia, western blotting was carried out in H460 NSCLC cells. The expression of ZNF746 was induced from 2 to 8 h in hypoxic H460 NSCLC cells (Fig. 4C). Of note, Twist, one of the EMT molecules, was also induced from 4 to 8 h in hypoxic H460 NSCLC cells almost in parallel with ZNF746.

Silencing of ZNF746 significantly suppresses the expression of Nanog and OCT4 at the mRNA level in H460 NSCLC cells. Although Nanog and OCT4 are transcriptional factors in embryonic stem cells (16), they have been studied in cell metastasis through the EMT process (17,18). In this regard, we determined the mRNA expression of OCT4 and Nanog by RT-qPCR following ZNF746 siRNA transfection. OCT4 and Nanog were significantly suppressed at the transcriptional level in ZNF746 siRNA-treated H460 NSCLC cells (Fig. 4D).

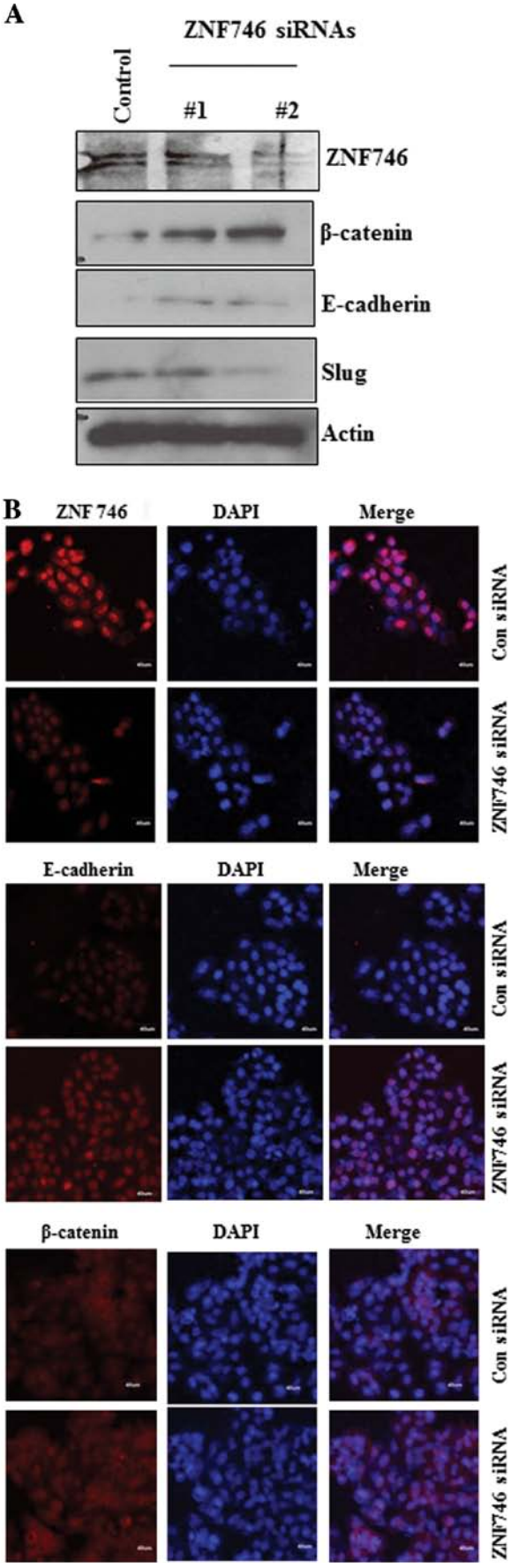

Figure 3. Silencing of ZNF746 upregulates E-cadherin and β-catenin in H460 NSCLC cells. (A) Effect of ZNF746 knockdown on ZNF746, E-cadherin, β-catenin, Slug and β-actin by western blotting. (B) Effect of ZNF746 knockdown on E-cadherin or β-catenin expression by immunofluorescence assay using confocal microscopy. Nuclei were stained with DAPI (scale bar, 40 μm).

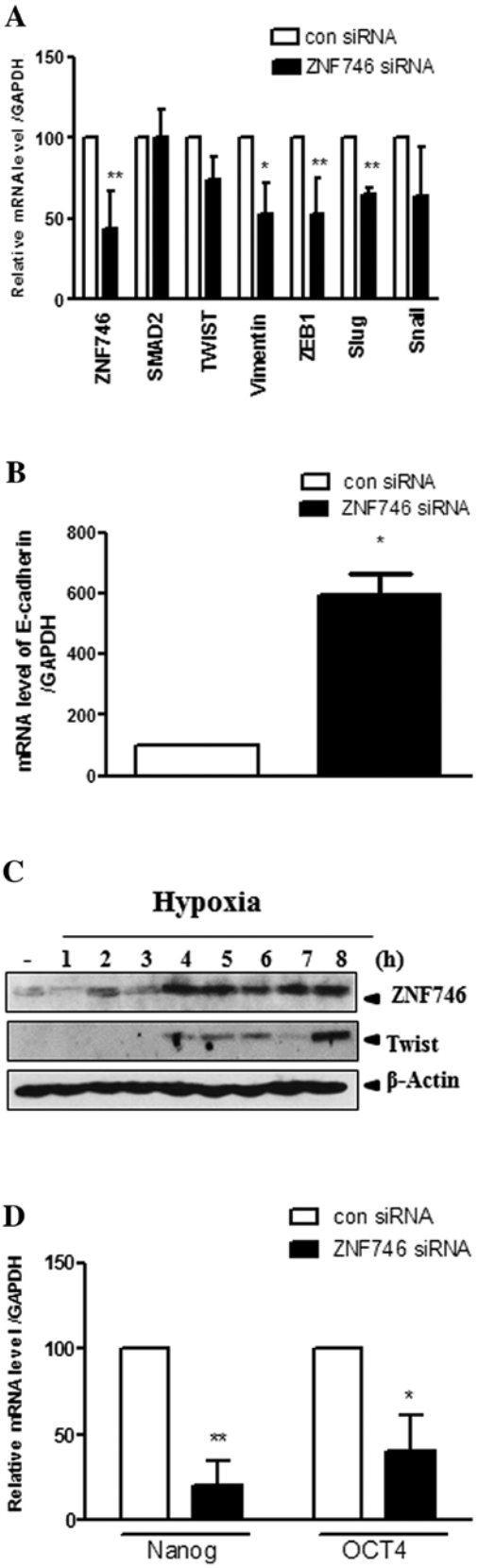

Figure 4. Silencing of ZNF746 upregulates EMT molecules at the mRNA level and hypoxia enhances the protein expression of ZNF746 in H460 non-small cell lung cancer (NSCLC) cells. Effect of ZNF746 knockdown on (A) ZNF746, SMAD2, Twist, vimentin, ZEB1, Slug, Snail and (B) E-cadherin at the mRNA level by real-time quantitative RT-PCR (RT-qPCR). (C) Effect of hypoxia on the expression of ZNF746 and Twist by western blotting. (D) Effect of ZNF746 knockdown on Nanog and OCT4 at the mRNA level by RT-qPCR and GAPDH was used for normalization. Data represent the means ± SD (N=3). *P<0.05 and **P<0.01 vs. siRNA control.

Discussion

Lung cancer is generally classified into small cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC). The lung is a common metastatic site from other parts of the body and primary lung cancer usually metastasizes to the brain, bones, liver and adrenal glands (19). Metastases represent the end products of a multistep cell-biological process known as the invasion-metastasis cascade in several types of cancer (20). In the present study, the role of ZNF746 was examined in the invasion and EMT in H460 NSCLC cells.

In the present study, invasion assay using Boyden chamber revealed that ZNF746 knockdown significantly reduced the number of the invaded H460 NSCLC cells. It is well documented that several MMP family members such as MMP1 (21), MMP2 (22), MMP7 (23) and MMP9 (24) are involved in invasion and metastasis. RT-qPCR analysis showed that the silencing of ZNF746 attenuated the expression of MMP1, MMP2 and MMP 9, but not MMP7, in H460 NSCLC cells, indicating that the ZNF746 knockdown can suppress the invasion of H460 NSCLC cells, since ZNF746 may mediate invasion-metastasis cascades.

Accumulating evidence suggests that EMT is a critical event in tumor invasion and metastasis (3,4,25). Thus, activation of the EMT is known to endow the invasive and metastatic properties upon cancer cells for the successful colonization of distal target organs (26). Previous studies also revealed that EMT is crucial in drug resistance to EGFR inhibitor in NSCLC (27) and promotes the cell invasion and metastatic property. NSCLC cells expressing epithelial phenotype are more sensitive to EGFR inhibition than NSCLC lines expressing mesenchymal phenotype such as vimentin (28-30). Here, western blotting showed that E-cadherin and β-catenin as epithelial phenotypes were upregulated, while Slug as a mesenchymal phenotype was downregulated in ZNF746 siRNA vector-transfected H460 NSCLC cells compared to untreated control, indicating that the silencing of ZNF746 inhibits invasion-metastasis cascades via regulation of EMT molecules. Accordingly, mRNA expression of E-cadherin was upregulated, while that of vimentin or Slug, Twist, ZEB was also attenuated at the mRNA level in ZNF746 siRNA-transfected H460 NSCLC cells, strongly demonstrating that the silencing of ZNF746 favorably regulates EMT molecules at the mRNA and protein levels in H460 NSCLC cells.

Hypoxia plays an important role in the progression and metastasis of cancer (31). Also, hypoxia inducible factor (HIF) α regulates transcriptional regulators of EMT such as Twist (32)and ZEB1 (33-35). In several cancer cell lines, N-cadherin, vimentin and fibronectin are upregulated in hypoxia exposure (32). Similarly, ZNF746 was induced by hypoxia in parallel with Twist expression in H460 NSCLC cells. In addition, several studies showed that transcriptional marker Nanog and OCT4 promoted malignancy and metastasis in lung adenocarcinoma (18), melanoma (16) and colorectal (17) cancer. Here, ZNF746 siRNA-transfected H460 NSLC cells suppressed the expression of Nanog and OCT4 in H460 NSCLC cells, indicating that ZNF746 knockdown may possibly regulate lung cancer progression and metastasis mediated by Nanog and OCT4.

In summary, the silencing of ZNF746 suppressed the invasion by inhibition of MMP1, MMP2 and MMP9, upregulated E-cadherin and β-catenin as well as downregulated Slug at the protein level, consistently enhanced E-cadherin and attenuated the expression of EMT key transcriptional factors such as vimentin, Slug, Twist and ZEB, suppressed the expression of Nanog and OCT4 in H460 NSCLC cells. Also, ZNF746 was induced by hypoxia in parallel with Twist expression in H460 NSCLC cells. Overall, our findings suggest that ZNF746 may be a critical target molecule in the invasion and EMT process in H460 NSCLC cells. Nevertheless, animal studies should be performed in the future to further validate our *in vitro* evidence.

Acknowledgements

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