Original Article
Silencing of ILT4 suppresses migration and invasion of non-small cell lung cancer cells by inhibiting MMP-2

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Abstract: Immunoglobulin-like transcript 4 (ILT4) has been shown to be highly expressed in different types of tumors and may play an essential role in promoting tumor progression. However, the effect of ILT4 on the invasive growth and metastasis of non-small cell lung cancer (NSCLC) and the underlying molecular mechanisms are not clear. In this study, high expression of ILT4 and MMP-2 was identified in clinical NSCLC tissues. In addition, the expression levels were closely associated with the clinical stage of NSCLC. MMP-9 was also highly expressed in NSCLC tissues, but showed no relationship with clinical stage on immunohistochemistry assay. The results of wound healing and transwell invasion assays further confirmed the role of ILT4 in the migration and invasion of NSCLC cells. The invasive and migratory capacity of A549 and H1299 cells was significantly suppressed after transfection with si-ILT4. Furthermore, silencing of ILT4 remarkably inhibited expression of MMP-2, but not MMP-9. Collectively, our results suggest that ILT4 may serve as a diagnostic biomarker and therapeutic target for patients with advanced or metastatic NSCLC.

Keywords: Non-small cell lung cancer, immunoglobulin-like transcript 4, migration, invasion, MMP-2, MMP-9

Introduction

Globally, lung cancer is the most prevalent tumor and is associated with high morbidity and mortality [1]. Non-small cell lung cancer (NSCLC) accounts for approximately 85% of all patients with lung cancer [2]. Most patients are asymptomatic until the middle and advanced stages and the 5-year survival rate is typically less than 15% [3-5]. Early detection and treatment is the key to improve the survival rate of patients with NSCLC as traditional therapies are often not effective in improving the survival of patients with advanced NSCLC [6]. Therefore, research on the expression of NSCLC genes, their functions and the underlying molecular mechanisms is a key imperative to develop molecular targeted therapies for lung cancer.

Tumor invasion and metastasis are complex processes; breaking through the extracellular matrix (ECM) is the initial and critical stage for dissemination and metastasis of tumor cells. Matrix metalloproteinases (MMPs) can effec-tively degrade almost all components of the ECM. MMPs can destroy the basement membrane, promote infiltration of tumor cells into surrounding normal tissues and eventually lead to tumor proliferation and metastasis [7]. Type IV collagen is the major structural component of the ECM and the basement membrane, while gelatinases (including MMP-2 and MMP-9) are the main enzymes that degrade type IV collagen [8]. Therefore, MMP-2 and MMP-9 play an essential role in tumor invasion and metastasis. High expression levels of MMP-2 and MMP-9 suggest a strong ability to break through the ECM, which is commonly seen in metastatic, advanced-stage and poorly-differentiated or highly malignant pathological subtypes of tumors. In a study by Herbst et al., low expression levels of MMP-2 and MMP-9 in NSCLC tissues were associated with improved survival and lower recurrence rate [9, 10].

Imunoglobulin-like transcript 4 (IL4) is a member of the ILT family and it is mainly expressed in monocytes, B cells and dendritic cell sarco-

ma (DCS) [11]. As an inhibitory receptor, ILT4 can combine with its corresponding ligands and recruit inhibitory molecules SHP-1 and SHP-2 through the immunoreceptor tyrosine-based inhibitory motif; this inhibits the activity of immune cells such as T cells, NK cells and mononuclear cells [12-14]. At present, few studies have investigated the expression of ILT4 and other ILT suppressor receptors in tumor cells. ILT3 and ILT4 were shown to be highly expressed in B-cell chronic lymphocytic leukaemia [15]. ILT4 was shown to be over-expressed in NSCLC, breast cancer, colorectal cancer and their corresponding cell lines. In addition, the expression levels showed a positive correlation with the numbers of tumor infiltrating lymphocytes in cancer tissues [16, 17]. Furthermore, the blockade of ILT4 in pancreatic cancer cells was shown to decrease cell motility [18]. High expression levels of ILT4 in different tumors and its related mechanisms indicate an important role of ILT4 in promoting tumor progression. However, the role of ILT4 in the invasion and metastasis of NSCLC and the underlying mechanisms are not yet clear.

Therefore, in the present study, the relationship of ILT4 with MMP-2 and MMP-9 was investigated, in addition to exploring the role of ILT4 in the invasion and metastasis of NSCLC and investigating the underlying mechanisms.

Materials and methods

Human tissue samples

The study was approved by the Ethics Committee of the Rizhao Hospital of Traditional Chinese Medicine. Clinical samples were collected from 30 patients with NSCLC from October 2015 to September 2017 after obtaining informed consent. All patients had a confirmed diagnosis of NSCLC, whereas none of the patients had received any cancer treatment. The clinical stage of NSCLC was classified as I-II or III-IV according to the World Health Organization classification and the pTNM staging system of the Union for International Cancer Control [19, 20].

Immunohistochemistry

Fresh NSCLC tissue samples were fixed in formalin for 48 hours, paraffin-embedded and cut into sections of desired thickness using a microtome. After washing, the samples were prepared for overnight incubation with antibod-

ies against ILT4, MMP-2, and MMP-9 diluted in 5% horse serum with chilled phosphate buffered saline at 4°C. Subsequently, the sections were incubated with diluted streptavidin-peroxidase HRP at room temperature with a staining kit, according to the manufacturer's instructions. The sections were then stained with haematoxylin for 5 minutes and examined under a phase-contrast microscope.

Cell lines and culture

Human NSCLC cell lines (A549 and H1299) were purchased from the Chinese Academy of Science Cell Bank (Shanghai, China) and routinely cultured in RPMI-1640 medium (Hyclone, USA) supplemented with 10% fetal bovine serum (FBS, Biowest, Barcelona, Catalonia, Spain) and 1% penicillin/streptomycin (P/S, Gibco, Grand Island, NY, USA) at 37°C in 5% CO_2.

Cell transfection

A549 and H1299 cells were cultured at a density of 1×10^6 cells/well into 6-well dishes. When the cells grew to 70%-80% confluence, siRNA against human ILT4 and the control siRNA synthesised by RiboBio (Guangzhou RiboBio Co., Ltd., Guangzhou, China) were transfected with lipofectamine 2000 Reagent (Invitrogen, USA) according to the manufacturer's protocol.

Wound healing assay

The transfected A549 and H1299 cells were seeded into 6-well plates and lightly scratched using a sterile pipette tip in the central axis of the plate when the density reached approximately 80%. After incubation for 24 and 48 hours, wound closure was monitored.

Transwell assay

The transfected A549 and H1299 cells were placed in the upper Transwell chamber (Corning Costar Corp, Corning, NY, USA) with insert pre-coated matrigel (BD, USA), and the medium containing 20% FBS was added to lower chamber. After incubation for 48 hours, the transfected cells that had migrated across the membrane were fixed, stained and counted.

Quantitative real-time PCR (qRT-PCR) assay

Total RNA was isolated from NSCLC cells using TRIzol Reagent according to the manufacturer's
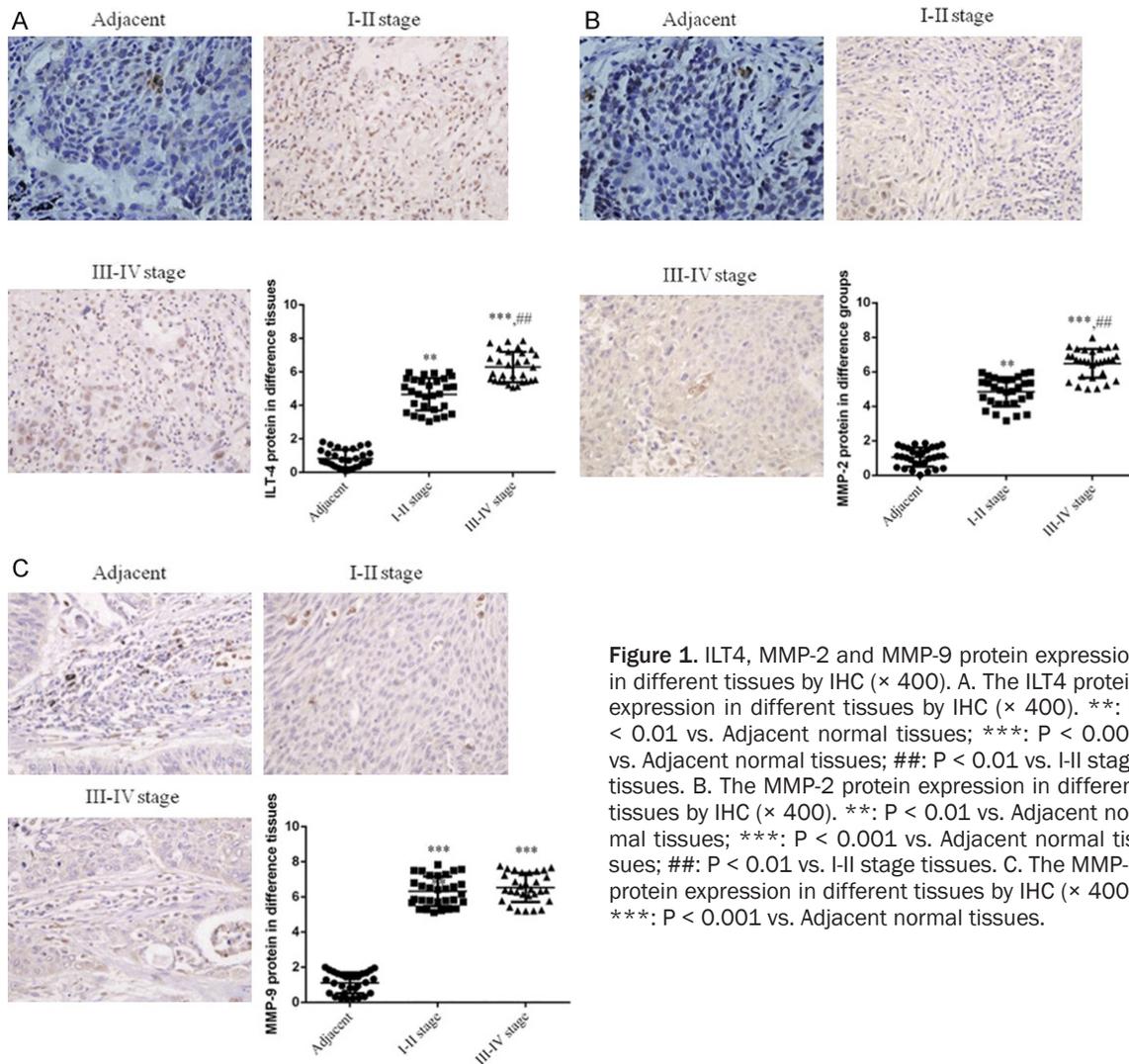
Figure 1. ILT4, MMP-2 and MMP-9 protein expression in different tissues by IHC (× 400). A. The ILT4 protein expression in different tissues by IHC (× 400). **: P < 0.01 vs. Adjacent normal tissues; ***: P < 0.001 vs. Adjacent normal tissues; ##: P < 0.01 vs. I-II stage tissues. B. The MMP-2 protein expression in different tissues by IHC (× 400). **: P < 0.01 vs. Adjacent normal tissues; ***: P < 0.001 vs. Adjacent normal tissues; ##: P < 0.01 vs. I-II stage tissues. C. The MMP-9 protein expression in different tissues by IHC (× 400). ***: P < 0.001 vs. Adjacent normal tissues.

instructions. qRT-PCR was performed to detect the relative mRNA expression levels of ILT4 in A549 and H1299 cells. The expression levels were normalized to those of the endogenous reference, GAPDH. The relative gene expression level was calculated using the comparative Ct method, and those relative to the calibrator were given by the formula 2-ΔΔCt.

Western blotting assay

NSCLC tissues and cell lines were collected and lysed in cold RIPA lysis buffer and the protein concentrations were measured using the BAC protein assay. All protein content was separated by 12% SDS-PAGE, transferred to PVDF membrane and then blocked with 5% non-fat milk with 0.1% Tween-20 (TBST) for 3 hours at room temperature. Subsequently, the membranes were incubated overnight at 4°C with

antibodies against MMP-2 (1:1000), MMP-9 (1:1000), ILT4 (1:1000) and GAPDH (1:500). Then the membranes were incubated with secondary antibodies coupled to horseradish peroxidase at room temperature. The protein bands were performed using an ECL Chemiluminescence kit. GAPDH was used as the internal control.

Statistical analysis

All experiments were performed in triplicate and data expressed as mean ± standard deviation (SD). Statistical analysis was performed using SPSS 18.0 software (SPSS Inc, Chicago, IL, USA). Between-group differences were assessed using one-way ANOVA and post hoc multiple comparisons were performed using the LSD method. P < 0.05 was considered indicative of statistical significance for all analyses.
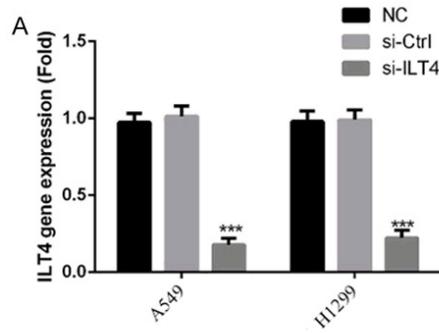
Figure 2. ILT-4 gene expression in different groups and cell invasion of different groups. A. ILT-4 gene expression in different groups. NC: normal control group; si-Ctrl: the cell transfected by empty vector; si-ILT4: the cell transfected by ILT4 inhibitor; ***: P < 0.001 vs. NC group. B. The invasion A549 cell of different groups by transwell assay. NC: normal control group; si-Ctrl: the cell transfected by empty vector; si-ILT4: the cell transfected by ILT4 inhibitor; ***: P < 0.001 vs. NC group. C. The invasion H1299 cell of different groups by transwell assay. NC: normal control group; si-Ctrl: the cell transfected by empty vector; si-ILT4: the cell transfected by ILT4 inhibitor; ***: P < 0.001 vs. NC group.
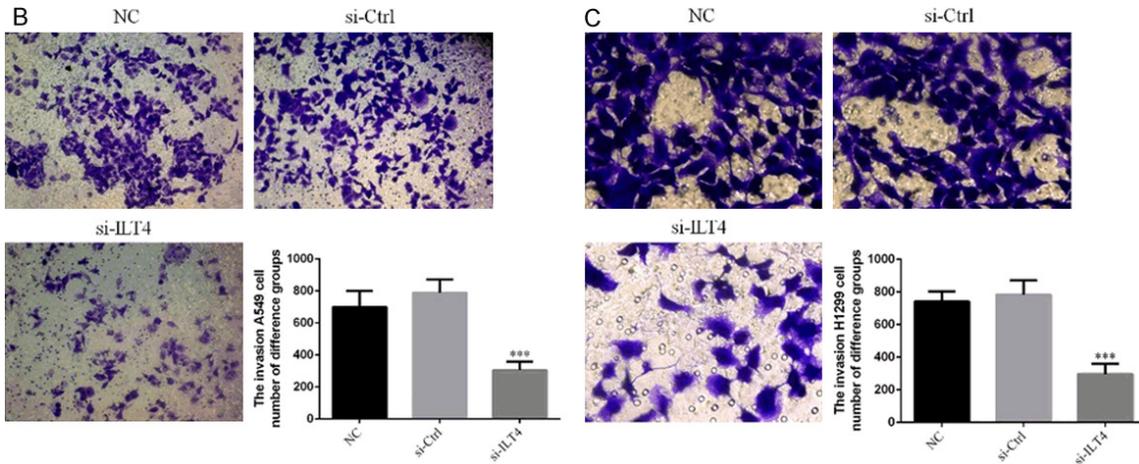
Results

Protein expression levels of ILT4, MMP-2 and MMP-9 in NSCLC tissues

The expression levels of ILT4, MMP-2, and MMP-9 protein in NSCLC tissues of patients with stage I-II disease were significantly greater than those in the anatomically contiguous normal tissues. Further, expression of ILT4 and MMP-2 in NSCLC tissues of patients with stage III-IV disease was higher than those in NSCLC tissues of patients with stage I-II disease. However, expression of MMP-9 in stage III-IV NSCLC tissues was not significantly different from that in stage I-II NSCLC tissues (**Figure 1**). These findings suggest that ILT4 is highly expressed in various stages of NSCLC and that ILT4 expression is associated with expression of MMP-2.

Effect of ILT4 on migration and invasion of NSCLC cells

The above results demonstrated the over-expression of ILT4 in NSCLC tissues and suggested its close relation with the development and progression of advanced NSCLC. Thus, the impact of ILT4 on the invasion and metastasis of NSCLC cells was further examined. si-ILT4 was transfected into A549 and H1299 cells and then post-transfection, expression of ILT4 in A549 and H1299 cells was remarkably suppressed (**Figure 2A**). Subsequently, wound healing assay was performed to evaluate the role of ILT4 in the migration of A549 and H1299 cells. The data demonstrated that depletion of ILT4 inhibited the migration of both A549 and H1299 cells (**Figure 2B**, **2C**). The results of transwell invasion assay showed similar results and that the invasive ability of A549 and H1299 cells was notably inhibited after transfection with si-ILT4 (**Figure 3A**, **3B**). The results indicate that silencing of ILT4 dramatically depressed the migration and invasion of A549 and H1299 cells. Expression of MMP-2 and MMP-9 in clinical samples of NSCLC was associated with prolonged survival rate and may be used as indicators of prognosis [9, 10]. To assess the relationship of ILT4 with MMP-2 and MMP-9, expression of MMP-2 and MMP-9 was analyzed in A549 and H1299 cells transfected with si-ILT4 using Western blotting assay (**Figure 4**). si-ILT4 transfection significantly inhibited gene and
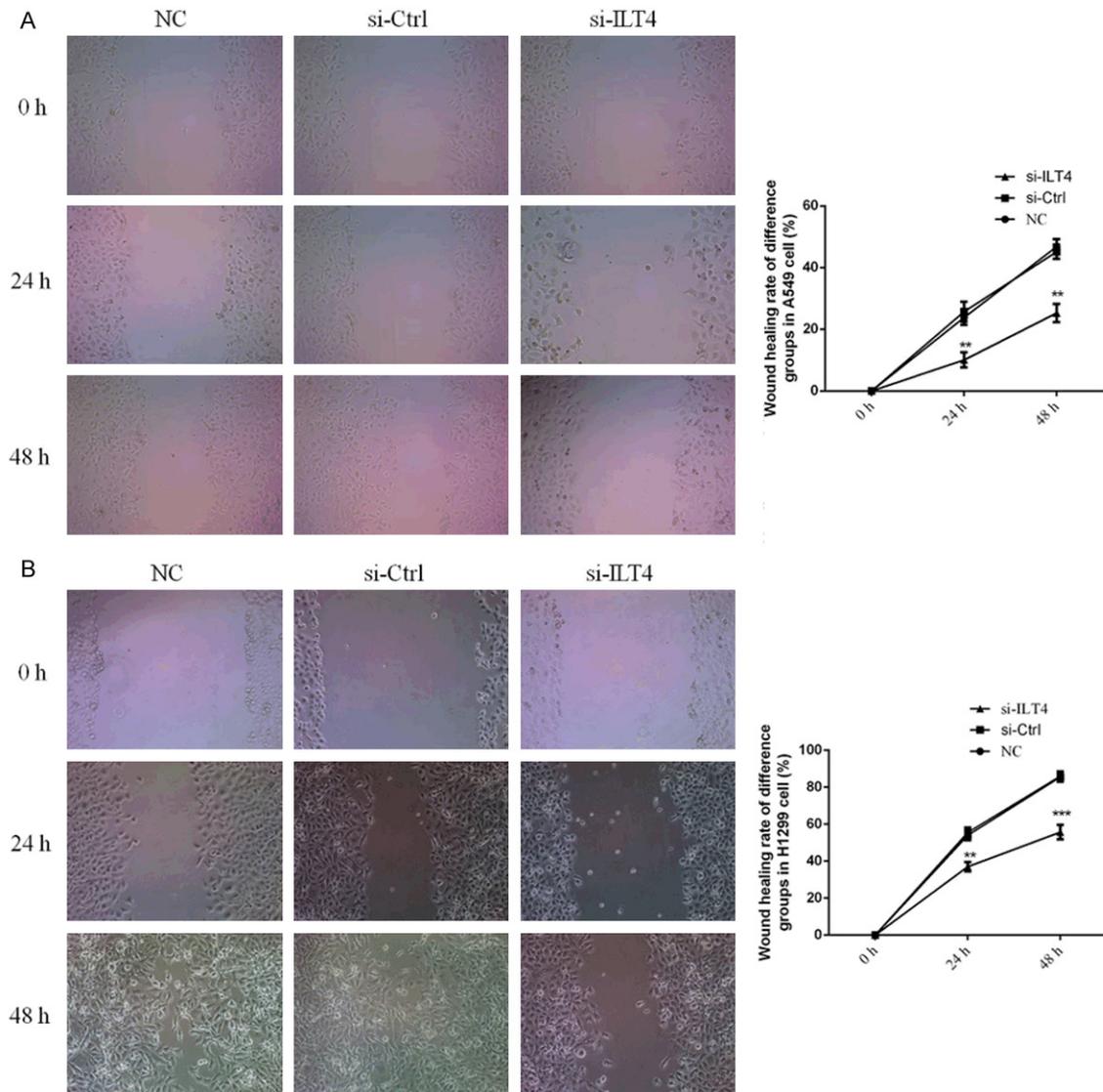
Figure 3. Cell migration of different groups by wound healing assay. A. The wound healing rate of different groups by wound healing assay in A549 cells. NC: normal control group; si-Ctrl: the cell transfected by empty vector; si-ILT4: the cell transfected by ILT4 inhibitor; **: P < 0.01 vs. NC group. B. The wound healing rate of different groups by wound healing assay in H1299 cells. NC: normal control group; si-Ctrl: the cell transfected by empty vector; si-ILT4: the cell transfected by ILT4 inhibitor; **: P < 0.01 vs. NC group; ***: P < 0.001 vs. NC group.

protein expressions of MMP-2, while it had no effect on the expression of MMP-9. These findings demonstrate that ILT4-knockdown likely suppressed the invasion and metastasis via inhibition of MMP-2.

Discussion

Lung cancer is one of the most malignant tumors that threaten human health and life across the world. The incidence of lung cancer and the associated mortality rates are increasing. Over the past year, more than 1 million

people worldwide have died of lung cancer [21, 22]. Recent years have witnessed considerable advances in the field of lung cancer treatment. However, the long-term survival rate of lung cancer patients continues to be poor and the overall 5 year survival rate is only 13%-15%, and the overwhelming majority of patients die from local recurrence or distant metastasis [23]. Invasive tumor growth and metastasis are the key factors that lead to poor prognosis of these patients. An estimated 70% of patients with lung cancer die of tumor metastasis. Even after radical resection of primary cancer, tumor
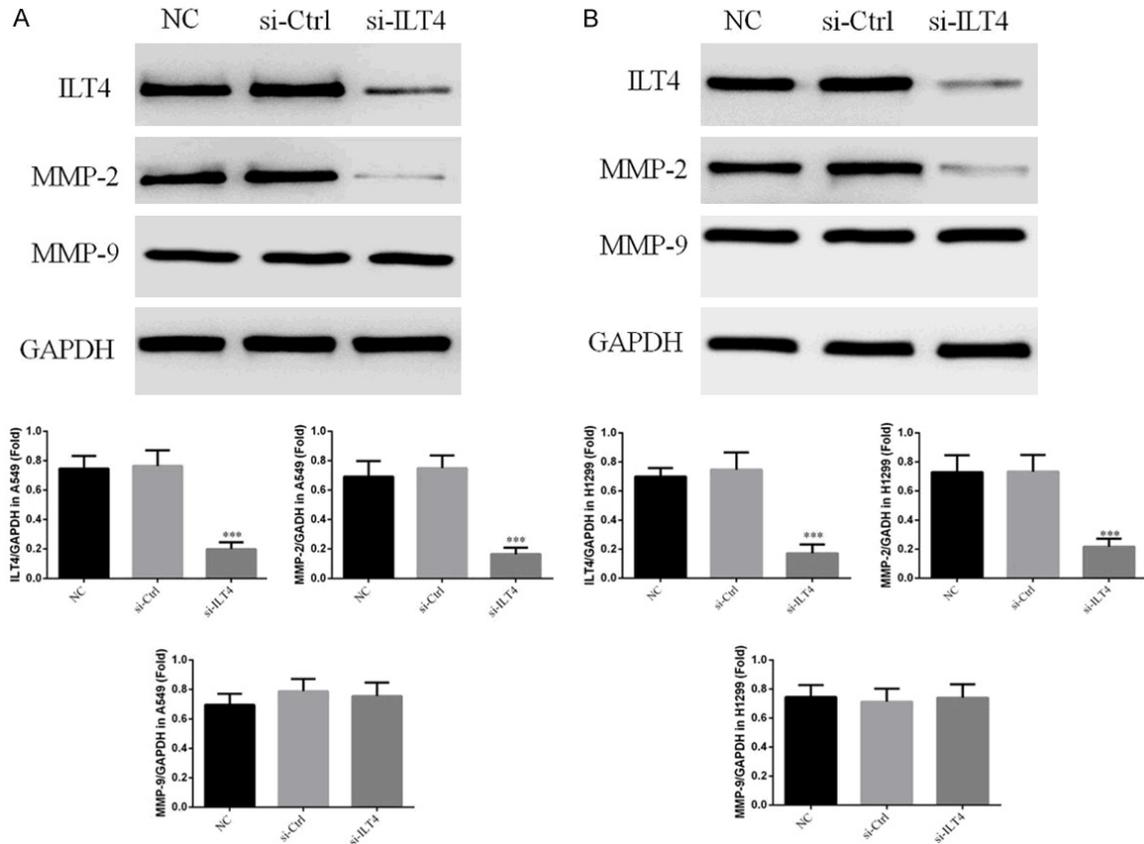
Figure 4. Relative protein expression of different groups by WB assay. A. The relative protein expression of different groups in A549. ***: P < 0.001 vs. NC group. B. The relative protein expression of different groups in H1299. ***: P < 0.001 vs. NC group.

metastasis is an important factor that affects the 5 year survival rate of patients with lung cancer. Although chemoradiotherapy has been widely used for treatment of metastatic lung cancer, its efficacy is not satisfactory [24, 25]. Therefore, elucidation of the molecular mechanisms of invasion and metastasis of lung cancer and identification of relevant molecular markers and therapeutic targets may facilitate early diagnosis and help improve treatment outcomes.

Tumor invasion and metastasis involve changes in tumor cells and tumor matrix components, including adhesion and migration of tumor cells and the degradation or destruction of ECM and the vascular basement membrane [26, 27]. ECM is a natural barrier to the infiltration and diffusion of tumor cells. The degradation of the ECM of adjacent tissues and penetration of the basement membrane and tissue microvasculature is an essential prerequisite for tumor cell infiltration and metastasis [28]. Degradation of

ECM mainly depends on the 4 types of protein hydrolases, i.e., serine proteinase, cysteine proteinase, aspartic proteinase, and matrix metalloproteinases (MMPs). MMPs are a group of zinc dependent endopeptidases which can degrade most components of the ECM [29]. Previous studies have shown that MMPs promote tumor invasion and metastasis by degrading the ECM and MMPs promote tumor growth and proliferation through angiogenesis [30, 31]. Among the MMPs secreted by tumor cells, MMP-2 and MMP-9 are the most important enzymes that degrade type IV collagen and play an important role in tumor infiltration and metastasis [32]. High expression of MMP-2 and MMP-9 has been demonstrated in lung cancer tissues and can be used as prognostic markers. Over-expression of MMP-2 and MMP-9 in NSCLC patients has been shown to be associated with poor prognosis [33, 34].

Immunoglobulin-like transcript 4 (ILT4) (also known as LILRB2, LIR-2, MIR10, or CD85d)

is an immunoglobulin-like transcription factor mainly expressed in monocytes and B cells. Dendritic cells (DC), NK cells, endothelial cells and placental cells exhibit low expression levels of ILT4 [35, 36]. ILT4 plays an immunosuppressive role in combination with classical or non-classical Major Histocompatibility Complex I (MHCI). Currently, research on the inhibitory receptor ILT4 has mainly focused on its influence on the function of immune cells and its potential use for promoting transplantation tolerance [37]. Expression of ILT4 in tumor cells is not well characterized. ILT4 can be expressed in NSCLC cells and can promote the progression of NSCLC through immunoregulation and non-immunomodulation via the ERK and PI3K/AKT/mTOR signaling pathways. In addition, ILT4 was shown to be co-expressed with the newly discovered angiopoietin-like proteins (ANGPTLs) and was closely associated with the clinical prognosis of NSCLC patients [38-41]. These findings highlight the need to further study the role of ILT4 in the development of NSCLC development.

In the present study, ILT4, MMP-2, and MMP-9 were highly expressed in NSCLC tissues; in addition, ILT4 and MMP-2 showed a close association with the NSCLC stage. This indicates that ILT4 plays an important role in the invasion and metastasis of NSCLC, which is likely mediated via regulation of MMP-2, but not MMP-9. Suppression of ILT4 significantly inhibited migration and invasion of NSCLC cells possibly via down-regulation of MMP-2 rather than MMP-9.

In summary, ILT4 was highly expressed in NSCLC tissues and was closely associated with the clinical stage of NSCLC. Silencing of ILT4 suppressed the migration and invasion of A549 and H1299 cells partially through down-regulation of MMP-2, but not that of MMP-9. These results indicate that ILT4 may serve as a diagnostic biomarker and therapeutic target for patients with advanced or metastatic NSCLC.

Disclosure of conflict of interest

None.

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