Knockdown of toll-like receptor 4 inhibits human NSCLC cancer cell growth and inflammatory cytokine secretion *in vitro* and *in vivo*

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Received March 12, 2014; Accepted May 14, 2014

DOI: 10.3892/ijo.2014.2479

Abstract. Toll-like receptor 4 (TLR4)-mediated signaling has been implicated in tumor cell invasion, survival and metastasis in several types of cancers. However, the expression of TLR4 in patients with non-small cell lung cancer (NSCLC) and its biological function in the development and progression of NSCLC have not been elucidated to date. Here, we sought to characterize the expression of TLR4 in patients with NSCLC and to investigate the biological roles of TLR4 in lung metastasis, cell invasion and survival. In this study, we found that TLR4 expression was elevated in most patients with NSCLC, and its expression levels correlated with key pathological characteristics, including tumor differentiation, stage and metastasis. Our data also showed that downregulation of TLR4 expression using an RNA silencing approach in A549 tumor cells significantly suppressed cell proliferation, cell migration and cell invasion, and induced tumor apoptosis *in vitro*, and suppressed tumor growth *in vivo*. In addition, we also found that downregulation of TLR4 expression significantly decreased cell TNF-α and IL-6 levels. Furthermore, we found that knockdown of TLR4 was able to significantly suppress constitutive phosphorylation of Akt and PI3K, which may contribute to the inhibition of tumor growth. These data suggest that TLR4 plays an important role in tumorigenic properties of human NSCLC, and that RNA interference-directed targeting of TLR4 could be used as a potential anticancer therapeutic target in NSCLC.

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Key words: non-small cell lung cancer, toll-like receptor 4, RNA silencing, cytokines

Introduction

Non-small cell lung cancer (NSCLC) accounts for approximately 85% of all lung cancers diagnosed (1), with less than 15% of patients surviving beyond 5 years due to lack of early diagnosis and effective treatment methods (2,3). Despite the advances in multimodal neoadjuvant chemotherapy and radiotherapy in treatment NSCLC, the outcome remains unsatisfactory because these therapies are toxic and almost never curative of metastatic NSCLC (4). Thus, this has highlighted the necessity for new therapeutic modalities, particularly for patients whose disease does not respond to conventional therapy.

Human toll-like receptors (TLRs) were first identified in mammalian immune cells, and belong to type I transmembrane proteins family consisting of an extracellular domain with a leucine-rich repeat region and an intracellular domain homologous to that of the human interleukin (IL)-1 receptor (5). TLRs have a powerful capacity to innate immune responses (6) through recognition of pathogen-associated molecular patterns (PAMP) expressed by viruses and bacteria, or host-derived PAMPs (7). Until now, 11 mammalian TLRs have been identified and found to be involved in the recognition of PAMPs (8,9). TLR-4 is an important member of type I transmembrane proteins family. Recently, growing evidence has shown TLR4 in various tumors (10-12), including head and neck, lung, gastrointestinal, liver, pancreatic, skin, breast, ovarian, cervical and prostate cancer (13). Although the TLR-4 profile varies in different tumor cells, current evidence indicates that the expression of TLR-4 and signaling cascade are involved in tumor growth, progression and invasion (14). For example, TLR-4 and signaling increased COX-2 and PGE2 signaling and early colorectal carcinogenesis, inhibited apoptosis and promoted angiogenesis (15). TLR4 increases Nanog gene expression, which induces liver oncogenesis (16). TLR4-mediated cancer growth involved in breast tumor progression and downregulation of TLR4 prevented breast cancer progression and survival (17). TLR4 expressed on human lung cancer cells is functionally active, and may play important roles in promoting immune escape of human lung cancer cells by inducing immunosuppressive cytokines and apoptosis resistance (18). TLR4 acts as a functional receptor in the pre-metastatic phase in pulmonary metastasis (19). These studies implied that TLR4 are widely expressed on human

tumor cells and may play important roles in the initiation and progression of cancer. However, to our knowledge, the effect of TLR4 expression on tumorigenicity in lung cancer *in vivo* and *in vitro* has been insufficiently reported (18,20), not fully clarifying the of TLR4 in NSCLC (20).

Therefore, in the present study we investigated the expression of TLR4 in NSCLC, and analyzed its association with the occurrence and development of NSCLC. In addition, we also developed a plasmid vector expressing siRNA-directed against TLR4 to investigate the key roles in tumor growth *in vitro* and *in vivo* for NSCLC.

Materials and methods

Tissue samples and clinical information. All patients gave written informed consent to participate in the study. This study was approved by the Ethics Committee of Jilin University, Changchun, Jilin Province, China.

Surgical tissue samples of human tumors and tumor-free tissues were obtained from patients undergoing surgical resection at the First Affiliated Hospital of Jilin University (Changchun, Jilin, China) from March, 2009 to July, 2013 after consent was obtained from the patients. All samples were immediately frozen in liquid nitrogen and stored at -80˚C until use. Tumor-free tissues were excised at least 5 cm away from the tumor border. Fifty tumor tissue and tumor-free tissues of NSCLC were classified into adenocarcinoma (24/50) and squamous cell carcinoma (26/50) according to the criteria of WHO (1997). Clinical information of all patients was collected including age, gender, smoking, tumor histological type, lymph node metastasis, TNM stage and differentiation. None of the patients received any prior radiochemotherapy. Clinical information of study subject is given in Table I.

Table I. The correlation of TLR4 expression with clinical pathologic features of NSCLC.

Clinical factor	Positive	Negative	P-value
Age			0.489
<60 (n=29)	18	11	
≥60 (n=21)	15	6	
Gender			0.356
Man (n=26)	17	9	
Women (n=24)	16	8	
Smoke			0.413
No (n=31)	20	11	
Yes (n=19)	13	6	
Metastasis			<0.01
No (n=20)	8	12	
N1-N2 (n=25)	10	15	
M1(n=5)	5	0	
Tumor differentiation			<0.01
Well (n=8)	1	7	
Moderate (n=26)	18	8	
Poor (n=16)	14	2	
TNM stage			<0.05
I-II (n=25)	8	17	
III-IV (n=25)	17	8	
Histological type			0.347
Adenocarcinoma (n=24)	15	9	
Squamous cell carcinoma (n=26)	18	8	

Immunohistochemistry. For immunohistological analyses, tissue specimens were fixed in 10% formalin buffer at pH 7.0 for 24 h and paraffin embedded. Lung tissue specimens were embedded in paraffin and cut into 3-μm sections for use in immunohistochemistry. Sections were dewaxed in xylene, rehydrated in alcohol in descending percentage, and blocked for endogenous peroxidase and avidin/biotin activities with 3% bovine serum albumin in 0.01 M phosphate-buffered saline (PBS, pH 7.2). Sections were incubated with mouse monoclonal antibody against human TLR4 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a dilution of 1:1,500 overnight at 4˚C. Samples were then washed in 0.01 M PBS (pH 7.2) for 5 min before incubation with biotin-labelled rabbit anti-mouse antibody (1:1,000; ZSGB-Bio, Beijing, China) for 2 h at 37˚C. After washing with PBS three times, the immunostain was visualized with a streptavidin-peroxidase reaction system (Wuhan Boster Biological Technology Ltd, Wuhan, China), and developed with diaminobenzidine hydrogen peroxide (Wuhan Boster Biological Technology Ltd).

The intensity of the staining was graded as: 0, if no immunoreactive cells were observed (negative); 1+, if the proportion of immunoreactive cells was <25%; 2+, if the proportion of immunoreactive cells was 25-75%; and 3+, if the proportion of immunoreactive cells was >75%. Values of 0 and 1 were considered to indicate negative staining, and 2 and 3 were considered to indicate positive staining.

Cell culture. The human non-small cell lung cancer cell line A549 was purchased from Cell Bank of Type Culture Collection of Chinese Academy of Sciences, Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). A549 cells were cultured in RPMI-1640 medium (Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA), cells were maintained at 37˚C in a humidified atmosphere containing 5% CO_2.

Construction of expressing plasmids. To inhibit the expression of TLR4, two short hairpin RNA (shRNA) targeting the TLR4 transcript were designed and synthesized and annealed. The synthesized oligonucleotides contain specific target sequence, a loop, the reverse complement of the target sequence, a stop codon for U6 promoter and two sticky ends. The target sequence in the oligonucleotide for suppressing TLR-4 was as followed: the siRNA1 sequence is: AACTTGTATTCAAGGTC TGGC (sense); the siRNA2 sequence is: AACTCCCTCCAGG TTCTTGAT (sense). The scramble sequence is: AATTCTCCG AACGTGTCACGT (sense). The empty vector (pCDNA-CMV) and scramble sequence had been tested in multiple cell lines and did not demonstrate any toxicity to cells as demonstrated by MTT assay after transfection, and had no effect on the expression of housekeeping genes, GAPDH or β-actin. The siRNA1, siRNA2 and scramble sequence were cloned into

expressing plasmid pCDNA-CMV, named as p-siRNA1, p-siRNA2 and p-scramble, respectively.

A549 cells were transiently transfected with empty vector (pCDNA-CMV), p-siRNA1, p-siRNA2 and p-scramble plasmid using Lipofectamine™ 2000 reagent (Invitrogen) according to the manufacturer's instructions, respectively. After 72 h of transfection, cells were collected and used for cell proliferation assays, cell apoptosis assays, western blot analysis, real-time quantitative PCR analysis, Matrigel invasion assay, caspase activity assay and cell inflammatory cytokines assay. Empty plasmid (pCDNA-CMV) acted as control group in the assays.

Quantitative PCR. Total RNA was isolated from A549 cell lines and NSCLCs tissue using RNeasy mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. RNA was prepared using the. RNA was reverse transcribed into cDNA by a Primescript™ RT reagent kit base on the manufacturer's protocols (Takara, Dalin, China). Quantitative real-time polymerase chain reaction (qPCR) assays were performed with SYBR-Green Real-Time PCR Master Mix (Toyobo, Osaka, Japan) and amplification equipment using specific primers: TRL4 forward, 5'-CGAGGA AGAGAAGACACCAGT-3' and reverse, 5'-CATCATCCTCA CTGCTTCTGT-3'; GAPDH forward, 5'-TGTGGGCATCAAT GGATTTGG-3' and reverse, 5'-ACACCATGTATTCCGGGT CAAT-3'. The PCR conditions were as follows: a pre-denaturing at 95˚C for 5 min, followed by 40 cycles of denaturation at 95˚C for 20 sec, annealing/extension at 55˚C for 20 sec, final extension 72˚C for 10 min. The amplification specificity was checked by melting curve analysis. The $2^{-\Delta\Delta CT}$ method (21) was used to calculate the relative abundance of target gene expression generated by Boineer Exicycler™ analysis software (Bioneer Corp., Daejeon, Korea). The expression of TLR4 were determined by normalization of the threshold cycle (Ct) of these genes to that of the control GAPDH. Each sample was run in triplicates.

Western blot analysis. After 72 h of transfection, A549 cells were washed twice with PBS and lysed in radio immune precipitation assay buffer for 30 min on ice. Cell lysates were clarified by centrifugation at 10,000 x g for 15 min, and protein concentrations were determined using the Bradford reagent (Sigma Chemical Co., St. Louis, MO, USA). Lysates were separated on 8 or 15% SDS-PAGE; proteins were transferred to Immobilon membrane (Millipore, Bedford, MA, USA) immunoblotted with specific primary antibodies and incubated with corresponding horseradish peroxidase- conjugated secondary antibody. Protein bands were visualized with enhanced chemiluminescence reagent (ECL, Amersham, GE Healthcare, Velizy-Villacoublay, France). The primary antibodies used in the western blots were as follows: antibodies against TLR4, β-actin, MMP-2 and MMP-9 (Santa Cruz Biotechnology); Akt, phosphorylated (p-) Akt, PI3K and p-PI3K (Sigma Chemical Co.). Secondary Abs used for immunodetection were: HRP-conjugated goat anti-mouse IgG and goat anti-rabbit IgG (Amersham Biosciences, Uppsala, Sweden).

MTT assay. The cell density of A549 cells was adjusted to $5x10^4$/ml, and cells were added to a 96-well plate (100 μl/well). In the blank controls, 100 μl of medium alone was added. At 24 h after culture, cells were transfected with the indicated plasmid. At 72 h after culture, 20 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT,5 mg/ml) (Sigma Chemical Co.) was added to each well followed by incubation at 37˚C for 48 h. Then, centrifugation was performed at 2,000 x g for 10 min. The supernatant was removed, and 200 μl of DMSO was added to each well followed by shaking for 10 min. Absorbance was measured at 570 nm test wavelength with an ELISA multi-well spectrophotometer (Molecular Devices Corp., Sunnyvale, CA, USA). The mean proliferation of cells without any treatment was expressed as 100%. Growth inhibition was calculated with the following formula (22): inhibition rate (%) =[1-(average absorbance of experimental group/average absorbance of blank control group)] x 100%.

Apoptosis analysis. A549 cells were cultured in 6-well plates in RPMI-1640 medium containing 10% FBS and were treated with indicated plasmid for 72 h. The cover slips were washed three times with PBS (pH 7.2), then cells were stained with 100 μg/ml acridine orange (AO) and 100 μg/ml ethidium bromide (EB) for 1 min. Cells were observed under a fluorescence microscope (Olympus, Tokyo, Japan). At least 200 cells were counted and the percentage of apoptotic cells was determined. We also detected caspase-3 and caspase-8 activity by ELISA as an additional indicator of apoptosis.

Caspase activity assay. The activity of caspase-3 and caspase-8 was measured using caspases colorimetric protease assay kits (Millipore Corporation, Billerica, MA, USA) according to the manufacturer's instructions. In brief, A549 cells were treated with indicated plasmid for 24 h. After treatment, cells were washed twice with ice-cold PBS (pH 7.2) and harvested by centrifugation at 700 x g for 10 min. The cell pellets were then lysed in 150 μl buffer provided in the kit. Protein concentrations of lysates were determined using the Lowry method (23). Then, an aliquot of lysates (80 μl) was incubated with 10 μl substrate of each caspase at 37˚C for 2 h. Samples were analyzed at 405 nm in a microplate reader (Thermo Fisher Scientific Inc., Waltham, MA, USA). The relative caspase activity of the control group was taken as 100. Each sample was run in triplicates.

Migration assay. Cell migration was determined using a scratch assay as previous described (24). In brief, the transfected cell lines were seeded on a 24-well plate and allowed to reach confluence. After scratching the bottom of the well with a pipette tip, then the monolayer of cells was washed three times with PBS (pH 7.2) to remove the detached cells. The remaining adherent cells were incubated in RPMI-1640 medium containing 1% FBS for 24 h; this medium was then replaced with RPMI-1640 medium containing 10% FBS. After 48 h, cell migration was evaluated using bright-field microscopy. The experiments were performed in triplicate.

Invasion assay. Invasion assay were performed using BD BioCoat™ Matrigel invasion chambers (Becton-Dickinson Labware, Bedford, MA, USA) according to the manufacturer's instructions. RPMI-1640 was added to the interior of the bottom and top chamber of inserts and allowed to hydrate for 2 h at 37˚C with 5% CO_2. Albumin at 100 ng/ml was added to the bottom chamber. Next, $5x10^4$ A549 cells transfected with

indicated plasmids were added to the top chamber of inserts and incubated at 37˚C and 5% CO_2 for 24 h. After incubation, cells at the bottom surface of the insert were fixed with purity methanol for 2 min, stained for 2 min in 1% toluidine blue supplemented with 1% borax (all from Sigma Chemical Co.), and rinsed twice with deionized water (distilled H_2O). The cells that had invaded to the lower side of the filter were observed under a Nikon phase-contrast microscope and counted in >10 fields of view at x200 magnification. The assay was done in triplicate. We also detected MMP-2 and MMP-9 by western blot analysis as an additional indicator of migration and invasion.

Human inflammatory cytokine assay. IL-6, IL-8 and TNF-α presence in the supernatant of transfected cells were detected according to the instruction of human inflammatory cytokine kit (BD™ Cytometric Bead Array, Becton-Dickinson Labware). FACScan flow cytometer (BD) was used to analyze samples.

Tumor xenograft assay. About 6-8 weeks old female BALB mice were obtained from the Institute of Laboratory Animal Science, Jilin University (Changchun, China), and were maintained under specific pathogen-free conditions and provided with food and water *ad libitum*. All animal experiments were performed in accordance with institutional guidelines, following a protocol approved by the Ethics Committees of the Disease Model Research Center, Jilin University (Changchun, China).

All the animals were fed with a normal pellet diet one week prior to the experimentation. A549 cells in exponential growth phase were harvested and single-cell suspensions ($2x10^6$ cells in 100 μl PBS) were injected subcutaneously (s.c.) into the right dorsal flank of nude mice. Tumor size was measured every 2 to 3 days, and tumor volume calculated as 0.5236 x width^2 x length. When tumors grew to an average volume of 75 mm^3, mice were randomly divided into siRNA, scramble group and control group (n=10 in each group), and inoculated with 30 μg/50 μl per mouse via i.t. injection of indicated plasmids one time a week for 21 days, respectively. Mice were sacrificed 7 days after the final plasmid injection. Tumor tissue was excised, measured volume and weighed. Some of the tissue was snap-frozen immediately for immunoblotting.

Statistical analysis. Statistical analyses were undertaken using the SPSS® statistical package, version 16.0 (SPSS Inc., Chicago, IL, USA) and the GraphPad Prism version 5.01 (GraphPad Software, San Diego, CA, USA) for Windows®. All data are expressed as mean ± SD. Statistical analysis between two samples was performed using Student's t-test. Statistical comparison of more than two groups was performed using one-way ANOVA followed by a Tukey's post hoc test. Pearson's correlation coefficients were used to determine whether two prognosis related factors were correlated to each other over all cases. P<0.05 was considered significant.

Results

TLR4 is upregulated in NSCLC and correlates with clinical features of patients with NSCLC. To identify the potential roles of TLR4 in the development and progression of NSCLC, we assessed its mRNA expression level and protein expression

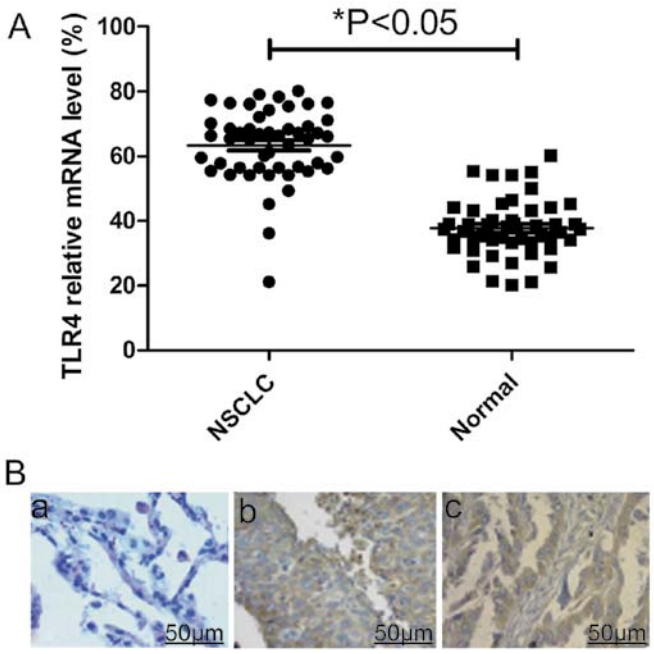

Figure 1. Levels of TLR4 are increased in NSCLC clinical samples. (A) Shown are quantitative real-time PCR results of relative expression level of TLR4 in 50 cases of NSCLC and cancer-free samples. The mRNA expression level of TLR4 was quantified as an internal standard and used to normalize the level of GAPDH from the same sample. (B) Shown are immunohistochemical results of TLR4 expression in NSCLC and matched cancer-free lung tissues: a, cancer-free lung tissue; b, adenocarcinoma; c, squamous cell carcinoma; *P<0.05 vs. cancer-free lung tissue.

level in 50 pairs of matched lung tissue samples by real-time polymerase chain reaction (qPCR) and immunohistochemistry, respectively. Real-time polymerase chain reaction (qPCR) assay showed that mRNA expression levels of TLR-4 were significantly higher in NSCLC tumors compared with their normal lung counterparts (P<0.05, Fig. 1A). At protein level, elevated levels of TLR4 protein were found in NSCLC tumors compared with the paired normal tissues from the same patients as shown by immunochemical staining (Fig. 1B).

Testing the association between TLR4 immunostaining with the clinicopathological parameters of the patients with NSCLC showed no significant differences with regard to patient gender, age and smoking history. The TLR4-positive tumors were of larger size, were poorly differentiated, had a higher TNM stage and were more likely to have metastasis than the TLR4-negative tumors (P<0.05, Table I).

Knockdown of TLR4 gene using siRNA in human A549 lung cancer cells. To study the biological role of TLR4 in NSCLC progression, we first constructed pcDNA3-CMV vectors expressing two small hairpin siRNA oligonucleotides targeting TLR4 (GenBank: NM-138554.3) to selectively reduce TLR4 gene expression in A549 cells, then the vectors expressing TLR4 siRNA or scramble siRNA were transfected into human lung cancer A549 cells. To determine the effect of siRNA on the endogenous expression of TLR4, the mRNA and protein levels of TLR4 were analyzed with real-time RT-PCR and western blot analysis, respectively. As shown in Fig. 2A, at mRNA level, there were different reductions in siRNA1, siRNA2 transfected cells and the decreased expression of TLR4 at mRNA levels

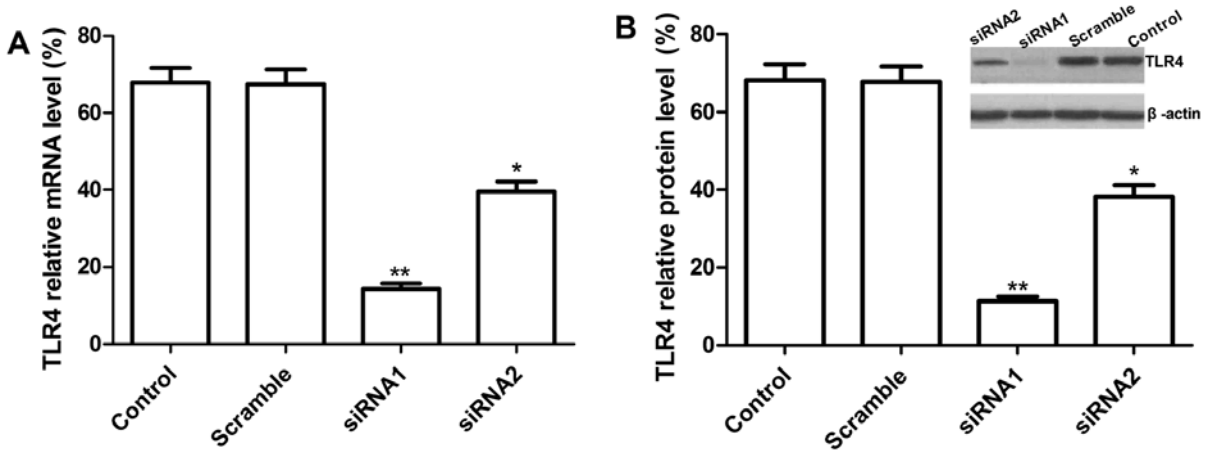

Figure 2. Knockdown of TLR4 inhibits the TLR4 expression in A549 cancer cells. (A) Quantitative real-time PCR analysis of TLR4 72 h after various plasmid transfection. (B) Western blot analysis of TLR4 72 h after various plasmid transfection and RNAi silencing; **P<0.01 vs. control.

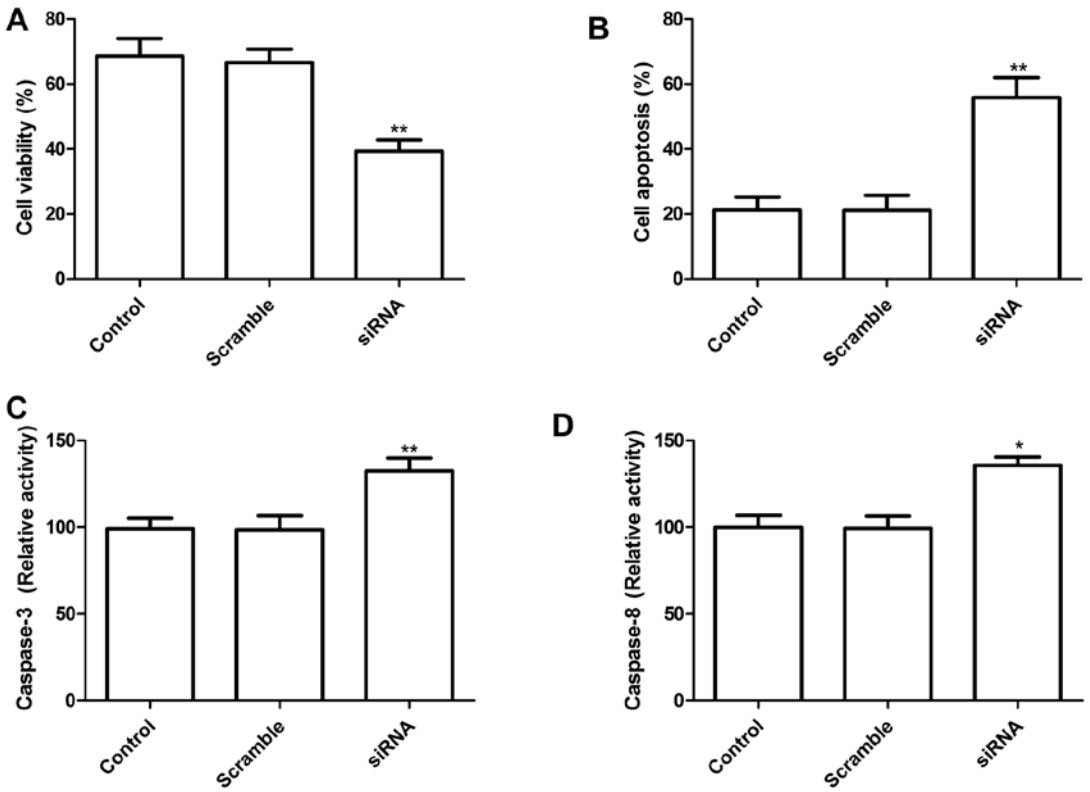

Figure 3. Knockdown of TLR4 inhibits cancer cell proliferation and induced cell apoptosis *in vitro*. (A) Cell proliferation and (B) cell apoptosis of A549 cells was determined after treated with indicated plasmid. (C) Caspase-3 and (D) caspase-8 activity of A549 cells was determined after treated with indicated plasmid *P<0.05, **P<0.01 vs. control.

for siRNA1, siRNA2 was 75.1±8.5, 46.2±4.7% as compared to vector control (P<0.05). However, no significant difference was observed in scramble and control group (empty group) (P>0.05). At protein level, western blot analysis results showed that two independent target sequences siRNA1 and siRNA2 markedly decreased protein expression of TLR4 compared with the scramble and control group (P<0.05, Fig. 2B) and siRNA1 had high reduction ratio as compared to siRNA2. Therefore, siRNA1 was chosen for use in subsequent functional assay.

Knockdown of TLR4 in lung cells reduces proliferation and induction of apoptosis. To determine the potential effects of siRNA-mediated TLR4 silencing on cell proliferation and survival, MTT analysis was performed 72 h after transfection with siRNA targeting TLR4. The results clearly show that transfection of A549 cells with siRNA targeting TLR4 significantly inhibited cell proliferation as compared to control group and scramble group (P<0.01, Fig. 3A). Next, the effects of the knockdown TLR4 by siRNA on lung cancer cell apoptosis were

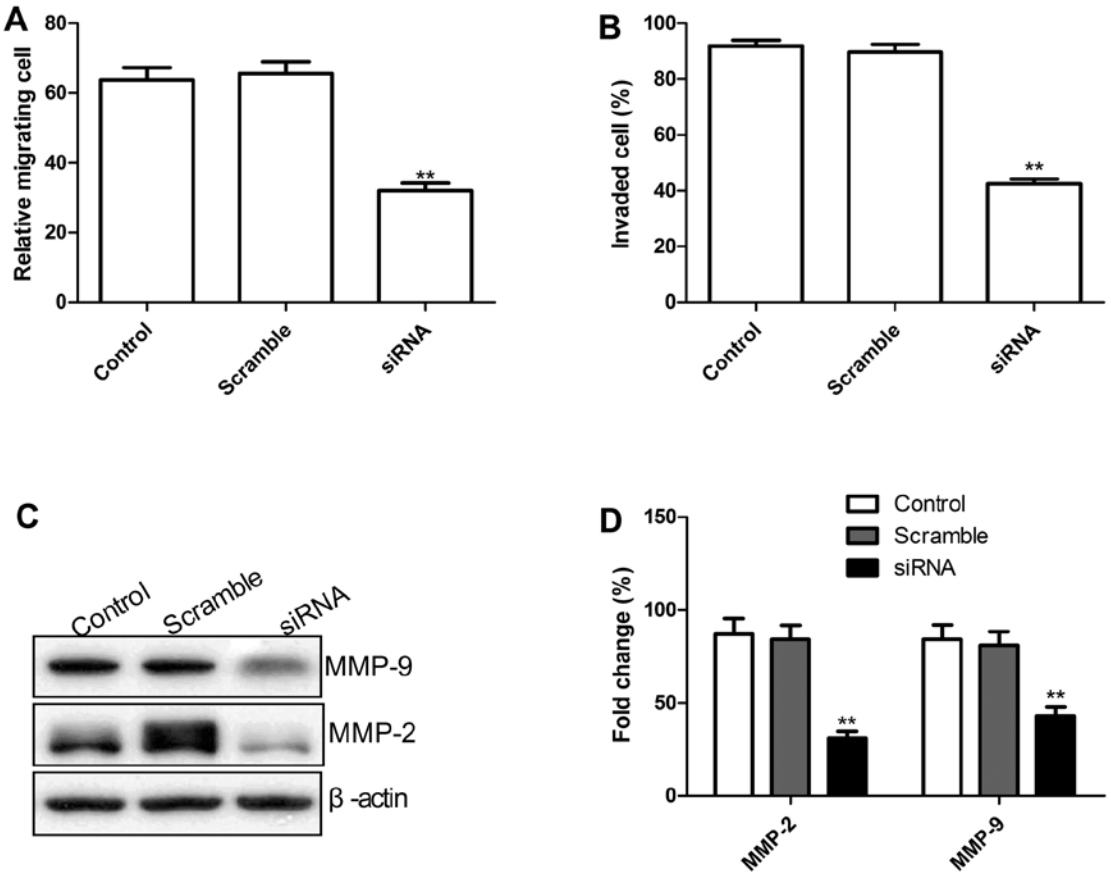

Figure 4. Knockdown of TLR4 inhibits cancer cell migration and invasion *in vitro*. (A) Cell migration and (B) cell invasion of A549 cells was determined after treated with indicated plasmid. (C and D) Western blot analysis was used to assess expression of MMP-2 and MMP-9 using antibodies against MMP-2 and MMP-9. β-actin was used as an internal control; $^{*}P<0.05$, $^{**}P<0.01$ vs. control.

assessed. As shown in Fig. 3B, knockdown of TLR-4 induced cell apoptosis compared to control group and scramble group (P<0.01).

To determine the potential mechanism of cell growth inhibition *in vitro*, caspase-3 and caspase-8 activity was detected using ELISA. Caspase-3 and caspase-8 activity was significantly decreased in knockdown TLR4 treatment groups, compared to the controls and scramble siRNA groups (P<0.05; Fig. 3C and D). These results suggest that reducing TLR4 levels may inhibit cell proliferation and induce cell apoptosis in lung cancer cells.

Knockdown of TLR4 in lung cells inhibits cell migration and cell invasion. To ascertain the inhibitory effect of knockdown of TLR4 on lung cancer on cell motility *in vitro*, scratch assay was performed to investigate their effects on the migration potential of A549 cells. As shown in Fig. 4A, knockdown TLR4 by siRNA significantly reduced the migration capacity in A549 tumor cells (P<0.01).

To evaluate the impact of the TLR4 knockdown on invasion of human lung cancer cells A549, invasion assay using the siRNA-transfected cells was performed. Our results showed that cell invasion ability in the knockdown TLR4 group was significantly decreased compared with controls and scramble groups, when assessed after 48 h by the modified Boyden chamber assays (Fig. 4B).

Migration and invasion play a crucial role in tumor metastasis. To determine the potential mechanism of the TLR4 knockdown on the inhibition of cell migration and invasion *in vitro*, MMP-2 and MMP-9 protein expression was examined using western blots. Western blot analysis displayed a significant decrease in MMP-2, and MMP-9 proteins in the knockdown TLR4 group infected A549 cells compared to control and Scramble groups (Fig. 4C and D). Taken together, these results suggest that reduction of TLR-4 on the inhibitory effect of metastasis of lung cancer was at least partially mediated by the downregulation of MMP-9 and MMP-2. These data indicate that TLR4 plays an important regulatory role in tumor cell migration and invasion.

TLR4 knockdown in lung cells inhibits TNF-α and IL-6. To examine the status of the TLR4-related inflammatory cytokines in the lung cell line A549 with TLR4 gene knockdown, ELISA assay was performed. Our results demonstrated that IL-6 and TNF-α were markedly depressed in the supernatant of silenced cells. The inhibition ration of cytokine IL-6 and TNF-α was 45.3±3.6 and 46.1±3.5%, respectively, when compared with vector control (P<0.05, Fig. 5A and B), no significant difference occurred in control group and scramble group (Fig. 5A and B). These results suggested that decreased TLR4 levels in tumor cells might reduce inflammatory cytokines.

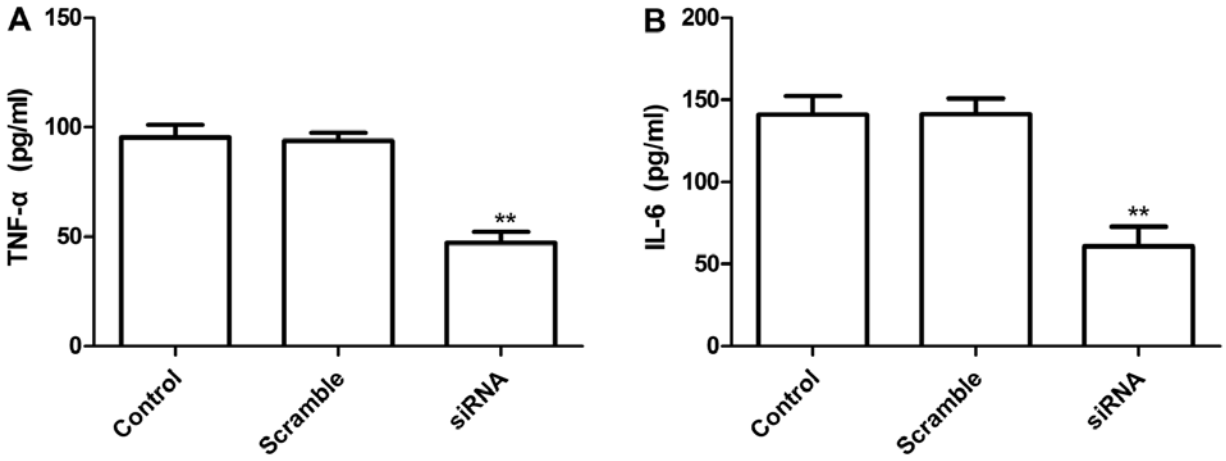

Figure 5. Knockdown of TLR4 inhibits inflammatory cytokines of A549 cells. (A) TNF-α and (B) IL-6 levels were measured by ELISA after treatment with indicated plasmid; *P<0.05, **P<0.01 vs. control.

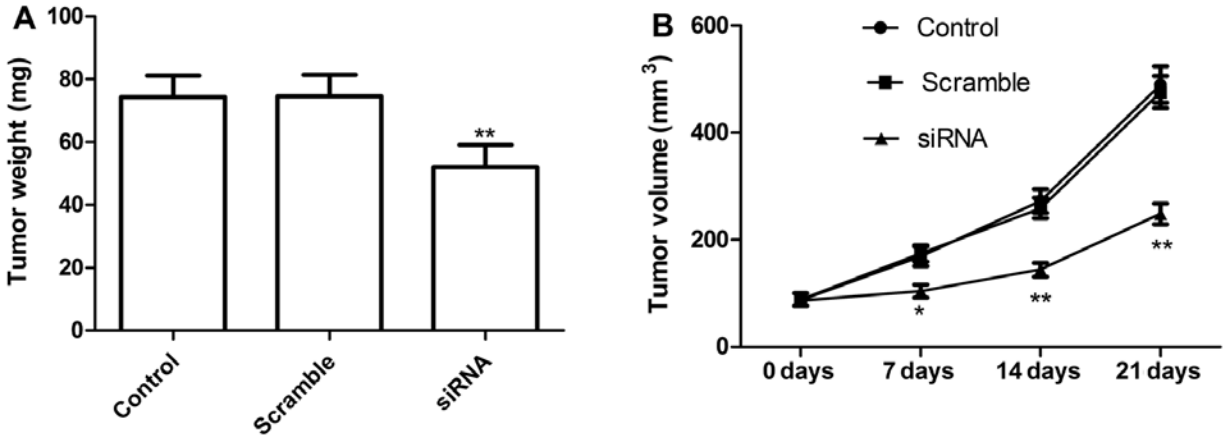

Figure 6. Knockdown of TLR4 inhibits lung tumor growth *in vivo*. (A) Tumor weight of mice 21 days after treatment with indicated plasmid. (B) Tumor volume of mice at days 7, 14 and 21. Data are expressed as the means ± SD; *P<0.05, **P<0.01 vs. control.

TLR4 knockdown in lung cells inhibits cell migration and cell invasion. We next determined if knockdown of TLR4 could inhibit tumor growth by a xenograft tumor model. At 7 days after the end of treatment, mice were sacrificed and tumor weights were measured. The tumor weight was significantly lower in the knockdown TLR4 group than in control and scramble groups (P<0.01; Fig. 6A). In addition, tumor volume also was determined at different time. Tumor volume in knockdown TLR4 group groups were significantly diminished when compared with the scramble and control group (Fig. 6B). These data demonstrated that knockdown of TLR4 suppressed tumor growth of NSCLC *in vivo*.

Effects of TLR4 on PI3K/AKT signaling pathway in A549 cells. To clarify the molecular mechanisms involved due to downregulation of TLR4 inhibition the growth of human lung tumor *in vitro* and *in vivo*, we focused on the effects of knockdown of TLR4 on the activation of PI3K/AKT signaling pathway, which participate in the main intracellular signaling pathway required for cell proliferation and survival. As shown

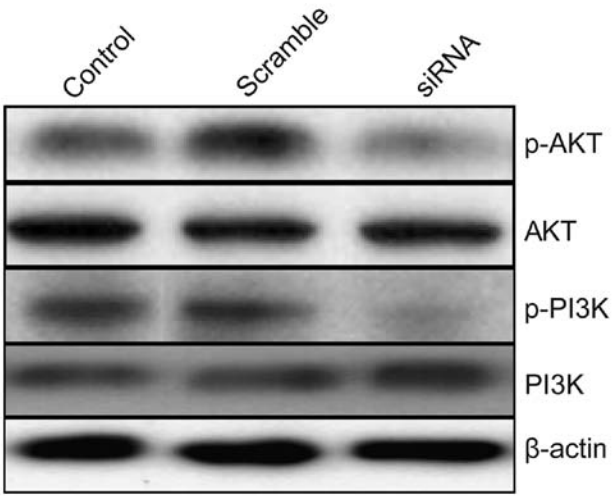

Figure 7. Knockdown of TLR4 inhibits PI3K/AKT signaling in A549 cells. Western blot analysis was performed using specific antibodies against the indicated proteins after treatment with indicated plasmid. Blots were reprobed for β-actin to normalize each lane for protein content.

in Fig. 7, compared with control group and scramble group, knockdown of TLR4 by siRNA resulted in a significant suppression of phosphorylation of Akt and PI3K. These results indicate that reduction of TLR4 by siRNA inhibits lung tumor cell growth, to some extent, by suppressing the PI3K/AKT signaling pathway.

Discussion

Lung cancer is one of the leading causes of cancer-related mortality worldwide (25). Lung cancer mortality rates have been rising in recent decades. It has been shown that chronic inflammatory disease is a risk factor for lung cancer (26). Since TLR-4 is also actively involved in the immune response against cancers, some researchers have suggested that TLR-4 exerts both a defensive role in normal cells and a negative role in cancer cells. However, the available evidence is still not conclusive on the link between TLR-4 and lung cancer. Bauer *et al* found that mutated TLR-4 in mice had less lung capillary permeability, less weight loss, leukocyte inflammation, and primary tumor formation (27), and that TLR-4 activation could protect the lungs from being inflamed during any potential tumorigenesis (28). This finding suggests the potential role of TLR-4 for airway inflammation and lung cancer progression. Yet, growing evidence has also found that TLR-4 is constantly expressed and upregulated in human lung cancer cells (18,20). He *et al* (18) found that the level of TLR-4 was significantly linked with the production of immunosuppressive cytokines, production of proangiogenic chemokine and with resistance to apoptosis by lung cancer cells. Zhang *et al* (20) showed that TLR4 expression was increased in patients with NSCLC, and TLR4 expression levels correlated with malignancy. In this study, our result showed that TLR-4 expression was elevated in most patients with NSCLC, and its expression level correlated with key pathological characteristics, such as, tumor differentiation, stage and metastasis, which was similar with previous results (20). These results imply that the presence of TLR4 in cancer cells may possess a negative role in lung cancer progression and metastasis.

A study on a larger number showed that TLR4 expression was increased in cancer cell or highly malignant tissues (20,29,30), and that activation of the TLR4 signaling transduction pathway promoted tumor progression and resistance to apoptosis (31). Hua *et al* showed that upregulation of TLR4 in human prostate cancer cells correlated positively with tumor metastasis (31). Kelly *et al* found that activation of the TLR4 signaling transduction pathway promoted tumor progression and chemo-resistance of epithelial ovarian cancer cells (11). Similar results were also observed in human head and neck squamous cell carcinoma and breast cell carcinoma where stimulation of TLR4 enhanced (29,32). In addition, growing evidence has shown that knockout TLR4 could inhibit cancer cell proliferation, cell metastasis and induce cancer cell apoptosis (11,17,29,32). Therefore, knocking down TLR4 from cancer cells could reduce tumor metastasis whereas stimulation of TLR4 on cancer cells would enhance the development of aggressive tumors. In the present study, we constructed pcDNA3-CMV vectors expressing two small hairpin siRNA oligonucleotides targeting TLR4 and transfected A549 cells to study the role of TLR4 in lung cancer and found that downregulation of TLR4 expression using RNA silencing approach in A549 tumor cells significantly suppressed cell proliferation, migration and invasion, and induced tumor apoptosis *in vitro*, and suppressed tumor growth *in vivo*.

An opinion that chronic inflammation promotes tumor development and progression has been supported by many epidemiological studies and experimental findings (33,34). It has been reported that when tumor cells are stimulated with lipopolysaccharides (LPS), a ligand for TLR4, the proinflammatory factors such as nitric oxide, IL-6 and IL-12 are expected to be released from tumor cells, attracting and activating inflammatory cells. Moreover, these factors play crucial roles in resistance of tumor cells to cytotoxic T lymphocyte (CTL) and natural killer cell (NKC) attack and facilitate evasion from immune surveillance (30). He *et al* found that knockdown of TLR4 *in vitro* lead to TLR4-related inflammatory cytokines being markedly depressed and so it could weaken the ability to the resistance of MDA-MB-231 to CTL and NKC attack and facilitate evasion from immune surveillance. In this study, our result showed that downregulation of TLR4 expression significantly decreased TNF-α and IL-6 levels, which was consist with previous results (18). These results may indicate that TLR4 knockdown *in vivo* inhibited the growth and promoted the death of lung tumors.

It has been reported that TLR-4 is involved in signal pathway regulation. It has been shown that the expression of higher levels of TLR-4 on human prostate adenocarcinoma (DU-145) cells and its activation, lead to NF-κB and proinflammatory cytokine production through the MyD88-dependent pathway (35). Another study showed TLR-4/MyD88-dependent signaling pathway involvement in laryngeal carcinoma progression (36). Hartmann *et al* showed that activated TLR-4 expression could enhance cancer cell growth, NF-κB translocation, and activated phosphatidylinositol 3-kinase/Akt pathway (37). Furthermore, He *et al* found that TLR4 activation contributes to active p38MAPK pathway, which is necessary for increased VEGF and IL-8 production (18). Hua *et al* study revealed evidence of a multifaceted signaling network operating downstream of TLR4-mediated tumor cell invasion, proliferation and survival (31). The phosphatidylinositol 3-kinase (PI3K)/AKT signaling pathway plays an important role in survival when cells are exposed to various kinds of apoptotic stimuli (38). Recent reports have indicated that the activation of Akt pathway is implicated in conferring resistance to conventional chemotherapy and multiple chemotherapeutic agents on cancer cells (39). Therefore, in the present study, we mainly focus on effect of TLR4 on the PI3K/AKT signaling pathway by western blot assay. Our results showed that downregulation of TLR4 expression using RNA silencing suppressed phosphorylation of Akt and PI3K, which indicate that TLR4 silencing inhibits tumor cell growth, to some extent, by suppressing activation of the PI3-K/Akt pathway signaling.

In conclusion, the present study demonstrated that TLR4 was elevated in most NSCLC and its expression level correlated with key pathological characteristics including clinical stage and metastasis and that knockdown of TLR4 could actively inhibit proliferation and survival of lung cancer cells *in vitro* and *in vivo*. Taken together, our results suggest RNAi-directed targeting of TLR4 may be a beneficial strategy for lung cancer therapy.

Acknowledgements

This study was supported by Science and Technology Research and Innovation Team funded of Jilin province (JL2013018).

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