Am J Transl Res 2017;9(6):2760-2774
www.ajtr.org /ISSN:1943-8141/AJTR0037384

*Original Article*

# Silencing of advanced glycosylation and glycosylation and product-specific receptor (RAGE) inhibits the metastasis and growth of non-small cell lung cancer

Yan Xia Yu, Wen Chong Pan, Yu Feng Cheng

*Cancer Treatment Research Center, Qilu Hospital of Shandong University, No.107, Wenhua West Road, Jinan, China*

Received August 5, 2016; Accepted January 20, 2017; Epub June 15, 2017; Published June 30, 2017

**Abstract:** Non-small cell lung cancer (NSCLC) constitutes the main cases of lung cancer and is the world's most common and lethal cancer owing to regional invasion or distant metastasis. Growing morbidity and lethality demonstrates that valid molecular target in management of NSCLC metastasis is still absence. The receptor of advanced glycation end-products (RAGE) has been identified as an oncogenic gene and appears to promote the growth and metastasis of various cancers. Here, we investigated if RAGE targeted by RNA interference (RNAi) might have certain effect on the restraint of the growth of NSCLC and tumor metastasis. Wound healing and Transwell invasion assays indicated that RAGE favored the metastatic capabilities of NSCLC H1975 cells. Besides, soft-agar colony assay revealed that silencing RAGE significantly blocked colony-forming capability of H1975 cells in vitro. Furthermore, we observed that RAGE participated in H1975 cells growth, metastasis and epithelial-mesenchymal transition (EMT) by regulating interdict crux intracellular signaling pathways, including phosphatidylinositol-3 kinase/serine-threonine kinase (PI3K/AKT) and V-Ki-ras2 kirsten rat sarcoma viral oncogene homolog/RAF proto-oncogene serine/threonine-protein kinase (KRAS/RAF-1). In xenograft model, significantly reduction intumor growth and Ki67 expression was demonstrated in nude mice inoculation with RAGE down-regulation H1975 cells. To conclude, our study demonstrated that RAGE played a crucial role in the metastasis and growth of NSCLC by regulating PI3K/AKT and KRAS/RAF-1 signaling pathways, thereby might be a promising therapeutic target for NSCLC.

**Keywords:** RAGE, NSCLC, metastasis, EMT

## Introduction

Non-small cell lung cancer (NSCLC) is one of the most common primary lung cancers andisa leading cause of death [1], despite a range of treatment options such as chemotherapy, clinical resection, and radiotherapy. Advanced and aggressive NSCLCs are highly invasive, metastatic, and resistant to treatment approaches. The most common sites of lung cancer metastasis are the lymph nodes, liver, adrenals, brain and bones [2]. For patients diagnosed in the terminal stages of NSCLC that present with metastatic, surgical tumor removal, chemotherapy, radiation or targeted therapies are all largely ineffective with a poor prognosis [3]. NSCLC cancer cells metastasis, the single most critical prognostic factor, is still poorly understood and a highly complex phenomenon. Consequently, searching for more effective therapeutic targets to combat NSCLC metastasis is crucial [4]. Central to this aim is a better understanding of the genetic changes and molecular events that occur during NSCLC growth and metastasis [5].

Abnormalities in the levels of several growth factor receptors such as epidermal growth factor receptor (EGFR) and the receptor for advanced glycation end-products (RAGE) are associated with different types of human cancers, including NSCLC [6]. RAGE, a member of the immunoglobulin super family, has been suggested to be involved in the survival, growth, and metastatic spread of various cancers and increased risk of multifarious malignant neo-

plasms [7]. RAGE is typically expressed at a low basal level in healthy tissue, although it is more highly expressed in lung tissue, implying a pulmonary function [8]. Recent studies proposed that up-regulation of RAGE is an important event in lung tumor development, and serum-soluble RAGE (sRAGE) serves as an effective, convenient, and sensitive diagnostic biomarker for lung cancer [9]. RAGE expression increases during tumorigenesis and progression of lung carcinogenesis, and has also been shown to play a part in the formation of primary lung neoplasms and progression to more advanced, aggressive lung adenocarcinomas, including NSCLC [10]. Investigation into the role of RAGE in tumor development indicate the involvement of certain intracellular signaling cascades, including phosphatidyl inositol 3-kinase (P13K), serine-threonine kinase (AKT), V-Ki-ras2 kirsten rat sarcoma viral oncogene homolog (K-RAS), and Mitogen-activated protein kinase (MAPK) [11]. These signaling pathways act cooperatively to control genes involved with various tumor cellular functions, including cell proliferation, survival, tumor angiogenesis, apoptosis, and metastasis [12]. Despite this research, the underlying mechanism of RAGE and its role in NSCLC growth and metastasis remain elusive, limiting targeted therapeutic options.

Owing to the likely involvement of RAGE in cancer development, we investigated the effects of targeting the gene in NSCLC metastasis. This showed that reducing expression of RAGE inhibited proliferation and suppressed cell invasion, metastasis, and migration in NSCLC cancer cells. In addition, we investigated possible molecular mechanisms and found that RAGE down-regulation blocked PI3K/AKT and KRAS/RAF-1 signaling pathways in NSCLC cells, thus decreasing NSCLC proliferation, epithelial-mesenchymal transition (EMT), and metastasis.

## Materials and methods

### *Cell lines*

The human non-small cell lung carcinoma cell lines H1975, A549, HCC827, H1650 and human bronchial epithelial cells BEAS-2B used as control were purchased from the Chinese Academy of Sciences Cell Bank of Type Culture Collection (CBTCCCAS, Shanghai, China). The NSCLC cell lines were maintained in RPMI-1640 or DMEM medium supplemented with 10% FBS, streptomycin (100 μg/mL) and penicillin (100 μg/mL). BEAS-2B cells was cultured in LHC-8 medium supplemented with 10% FBS. All cells maintained in an incubator with a humidified atmosphere containing 5% $CO_2$ at 37°C.

### *RNA interference*

RAGE siRNA was synthesized by Biomics Biotechnologies co. Ltd (Shanghai, China). H1975 cells were transfected with RAGE siRNA (sense, 5'-GACCAACUCUCUCCUGUAUTT-3' and antisense, 5'-AUACAGGAGAGAGUUGGUCTT-3' or non-specific siRNA (0.5 μg/well for 96 well plates and 2 μg/well for 6 well plates) using the Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations. After 48 h post-transfection, H1975 cells were used for western blot, clonogenicity, invasion, migration and proliferations assay [13].

### *Cell proliferation assay*

Cell viability was determined by the CellTiter 96® AQueous one solution cell proliferation assay (MTS) (Promega). H1975 cells was seeded in 96-well cell culture plastic plates at a density of $1 \times 10^6$ cells/well. After incubation for 24 h, 48 h or 72 h, respectively, 20 μl of One Solution reagent was added to each well and incubation was continued for additional 4 h. The absorbance was measured at 490 nm using Synergy™ HT Multi-Mode Microplate Reader (Bio-Tek, Winooski, VT, USA) [14].

### *Colony formation assay*

H1975 cells colony formation assay was performed to measure the growth ability of single H1975 cell to grow into a colony in vitro. Briefly, after transfecting H1975 cells with RAGE siRNA or scrambled control for 48 h, the transfected H1975 cells were seeded in complete RPMI-1640 media at a density of $2 \times 10^4$ cells in 60 mm dishes, which containing a top layer of 1% agar and a bottom layer of 10% agar. The plates were incubated for 4 weeks at 37°C and then stained with 0.1% crystal violet. Colonies with greater than 50 cells were counted manually. For clonogenicity assay, H1975 cells ($1 \times 10^3$) were plated onto a 6-well tissue culture plate in

1640-medium and incubated at 37°C. Cells were allowed to grow in complete medium for 5 days. Then cells were counted under the microscope. Five random fields were counted under a light microscope [15].

*Wound healing analysis*

Cell migration ability of RAGE silenced H1975 cells were detected by the scratch assay. After RAGE siRNA transfection, H1975 cells were seeded in 6-well plastic plates at the density of $3 \times 10^5$ cells/well. After 24 h, H1975 cells reached 90-100% monolayer confluence. A straight scratch was artificial created in the cell monolayers with 100 μl sterile pipette tip. Cells debris by the scratch was removed with phosphate buffer saline (PBS) and cultures were then supplemented with fresh 1640 medium for 48 h at 37°C. Migration images were captured using an inverted microscope (CarlZeiss, Hallbergnoos, Germany). The scratch wound widths were measure under microscope and the relative percentage of wound closure was determined by comparing to control cells [16].

*Migration assay*

The effect of RAGE siRNA on H1975 cells migration was evaluated by Transwell migration assay. After RAGE siRNA transfection for 48 hours, 200 μl $5 \times 10^3$ H1975 cells were plated into the upper chamber of the Transwell chamber with a non-coated polycarbonate membrane (8.0 μm, Corning). Total 600 μl RPMI-1640 medium containing 10% FBS was added to the Transwell lower chamber. After incubation for 6 h, an H1975 cell on the lower surface of polycarbonate membrane was fixed with 4% paraformaldehyde and was stained with 0.1% crystal violet. The non-migrated H1975 cells on the upper-side of the polycarbonate membrane were removed with a cotton swab. Image of the H1975 cells which migrated to the under-surface of the membrane was captured at 5 different microscopic fields. The number of migrated H1975 cells was counted from five randomly fields per polycarbonate membrane [17].

*Invasion assay*

$2 \times 10^4$ H1975 cells was plated into the top-chamber of the Transwell with Matrigel-coated (1 mg/ml, BD Matrigel™) polycarbonate membrane and allowed to invasive for 6 h. Total 600 μl RPMI-1640 medium with 10% FBS was added to the Transwell lower chamber as a chemo-attractant. H1975 cells remaining on top of the polycarbonate membrane were removed with a cotton swab. The invasive cells were fixed in 4% paraformaldehyde and the polycarbonate membrane was mounted in Hoechst 33258. Invasion cells were analyzed using fluorescence microscopy and the number of invasion cells was counted from five randomly selected fields per polycarbonate membrane [18].

*Gelatin zymography*

After siRAGE transfection, the medium was changed to serum-free RPMI 1640. H1975 cells were cultured for 24 h, and the conditioned media were collected. The media were electrophoresed on 10% precast polyacrylamide gels containing 0.1% gelatin (Sigma). The enzymatic activity of MMP-2 and MMP-9 was measured using a gelatin zymography kit (Life Technology) according to the manufacturer's protocol [19].

*MMP-2/9 activity analysis*

The activity of MMP-2/9 was determined by QuickZyme MMP-2/9 activity assay (QucikZyme BioSciences) according to the manufacturer's instructions. Briefly, after RAGE siRNA transfection for 48 h, H1975 cells were washed with fresh medium and replaced with serum-free medium. After 24 h, the medium was collected and centrifuged at 10000 g for 10 minutes. The cell supernatant was added to the 96-well strip which previous coated with MMP-2/9 antibody and incubated at 4°C overnight. After washing with wash buffer for 3 times, 50 μl assay buffer was added into the well, followed by adding 50 μl detection reagent. After incubation at 37°C for 1 h, OD405 was measured with Synergy™ HT Multi-Mode Microplate Reader (Bio-Tek, Winooski, VT, USA) [20]. The relative activity of MMP-2/9 was determined by comparing to H1975-Scr cells.

*Immunocytochemistry*

H1975or BEAS-2Bcells were seeded on glass coverslips. After 24 h, they were fixed by pre-cold acetone, then rinsed three times with PBS. The cells were permeabilized in 0.1% Triton X-100 and incubated with 1% BSA/PBS to block

nonspecific binding. Subsequently, the cells were immunostained by incubating with rabbit monoclonal antibody against RAGE (diluted 1:500, Epitomics) overnight at 4°C. After being washed with PBS, cells nuclei were counterstained with Mayer's hematoxylin solution (Amber Scientific, Midvale, WA). Images were taken and analyzed using the ZEN 2011 imaging software on a Zeiss invert microscope (CarlZeiss, Hallbergnoos, Germany) under 200-fold magnification.

*Western blot analysis*

H1975 cells were transfected with RAGE siRNA for 48 h. Whole-cell lysates were prepared with RIPA buffer containing protease and phosphatase inhibitors. Equal amounts of cell lysates (30 μg) were loaded on 10% SDS-PAGE and transferred onto PVDF membranes. After membranes were blocked, they were incubated with monoclonal antibody against RAGE (1:1000, Signalway Antibody), AKT (1:500, Signalway Antibody) and p-AKT Ser473 (1:10000, Epitomics), p-mTOR Ser2448 (1:5000, Epitomics) and phosphor-PI3K Tyr458 (1:1000, Cell Signaling Technology), PI3K (1:1000, Cell Signaling Technology), mTOR (1:1000, Cell Signaling Technology), p-p70s6k Ser371 (1:1000, Cell Signaling Technology), p70s6k (1:1000, Cell Signaling Technology), p-KRAS Ser181 (1:1000, Cell Signaling Technology), p-RAF-1 Ser289 (1:1000, Cell Signaling Technology), Twist (1:1000, Cell Signaling Technology), E-cadherin (1:1000, Cell Signaling Technology), N-cadherin (1:1000, Cell Signaling Technology), Slug (1:1000, Cell Signaling Technology) and GPADH (1:5000, Bioworld Technology) followed by incubation with goat anti-rabbit IgG (H&L) HRP (1:10000, Bioworld Biotechnology). All target proteins were detected by the ECL Gel system (Millipore, Braunschweig, Germany) and were visualized with the ChemiDoc XRS system (Bio-Rad, Hercules, CA, USA) [21].

*Quantitative real-time PCR*

Total RNA was isolated using the TRIzol reagent (Invitrogen) according to the manufacturer's recommendations. The cDNA was then synthesized with 1 μg total RNA using a PrimeScript RT reagent kit (TakaraBio) according to the protocol. QRT-PCR was performed using IQTM SYBR Green supermix and the iQ5 real-time detection system (Bio-Rad Laboratories). The comparative cycle threshold (Ct) method was applied to quantify the expression levels through calculating the $2(-^{\Delta\Delta}Ct)$ method. The primers used for PCR were as follows (sense and antisense, respectively): GAPDH: 5'-TGTGGGCATCAATGGATTTGG-3' (forward) and 5'-ACACCATGTATTCCGGGTCAAT-3' (reverse); RAG: 5'-GTGTCCTTCCCAACGGCTC-3' (forward) and 5'-ATTGCCTGGCACCGGAAAA-3' (reverse); RAF-1: 5'-GGGAGCTTGGAAGACGATCAG-3' (forward) and 5'-ACACGGATAGTGTTGCTTGTC-3' (reverse); KRAS: 5'-ACAGAGAGTGGAGGATGCTTT-3' (forward) and 5'-TTTCACACAGCCAGGAGTCTT-3' (reverse); mTOR: 5'-ATGCTTGGAACCGGACCTG-3' (forward) and 5'-TCTTGACTCATCTCTCGGAGTT-3' (reverse); AKT: 5'-AGCGACGTGGCTATTGTGAAG-3' (forward) and 5'-GCCATCATTCTTGAGGAGGAAGT-3' (reverse); PI3K: 5'-GGCGAAACGCCCATCAAAAA-3' (forward) and 5'-GACTCCCGTGCAGTCATCC-3' (reverse); p70s6k: 5'-CGGGACGGCTTTTACCCAG-3' (forward) and 5'-TTTCTCACAATGTTCCATGCCA-3' (reverse). The GAPDH RNA expression was used to normalize the target genes levels.

*Xenograft tumor assay*

Female BALB/c nu/nu mice (4-6-weeks-old) were obtained from Shanghai Slack laboratory animal co., LTD and maintained in specific pathogen-free conditions. All experimental protocols were approved by the Committee for the Care and Use of Laboratory Animals of People's Hospital of Xinjiang Uygur Autonomous Region and all experiments were performed in accordance with the approved guidelines and regulations. For the subcutaneous tumor model, H1975 cells transfected with RAGE siRNA or control siRNA suspended with PBS at a concentration of $1 \times 10^5$ cells/μl. A volume of 0.1 ml H1975 cells was subcutaneously injected into the right flank of anesthetized mice (n = 3 for each group) and the mice were observed for 4 weeks. The xenograft tumor volume was measured every 3 days and calculated as $(W^2 \times L)/2$, where W and L refer to the shorter and longer dimensions of the tumor, respectively. The H1975 tumor specimen were fixed in 10% formalin, embedded in paraffin and sectioned at 5 μm. For immunohistochemical assay, endogenous peroxidases were blocked in 3% hydrogen peroxide. Immunohistochemistry for Ki67, p-PI3K, p-AKT, p-mTOR, p-KRAS, p-RAF-1 and p-p70s6k were carried out on tumor tissue sections using Biogenex IHC detection system

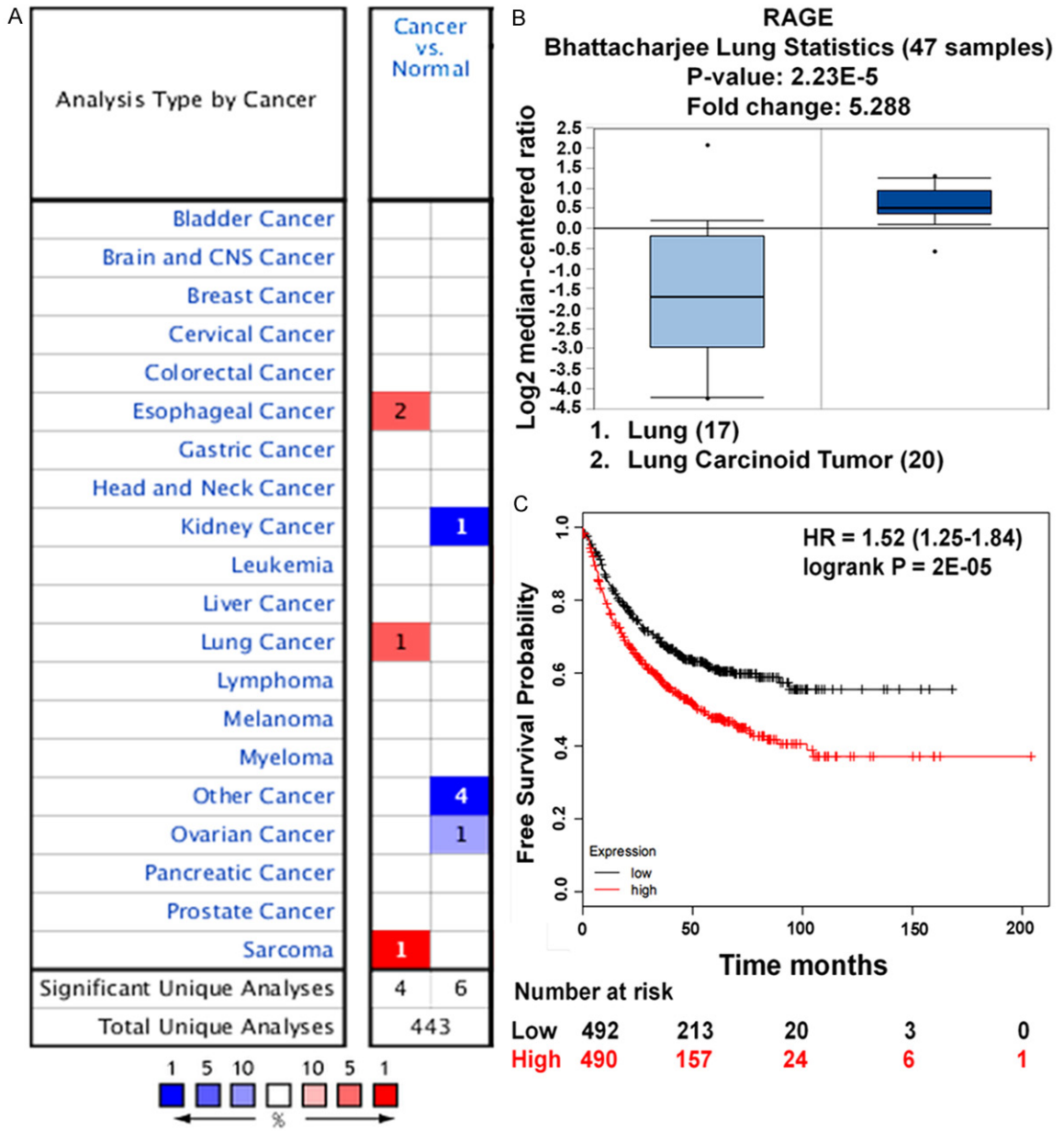


**Figure 1.** RAGE expression in various tumor types. A. The graphic compared the number of datasets that had RAGE over-expression (red, left column) and RAGE under-expression (blue, right column) in cancer tissue versus normal tissue. B. RAGE analysis in lung cancer (Oncomine database). Box plots derived from Bhattacharjee lung statistics database in Oncomine comparing expression of RAGE in normal tissue (left plot) and lung cancer tissue (right plot). P = 2.23E-5. Fold change = 5.288. C. Kaplan-Meier plots analysis of overall survival in lung cancer stratified by the expression of RAGE. In red: patients with high expression of RAGE and in black, patients with low expression of RAGE gene. P = 2.E-05. The *P* value was calculated by the log-rank test.

according to manufacturer's guidelines with anti-Ki67 antibody [22].

*Statistical analysis*

The data were presented as mean ± SD. The differences between groups were evaluated by either two-tailed Student's t test or one-way ANOVA followed by post hoc Dunnett's test. The GraphPad Prism 5 software was used to do the statistical calculations. Probability values of 0.05 were considered as statistically significant.

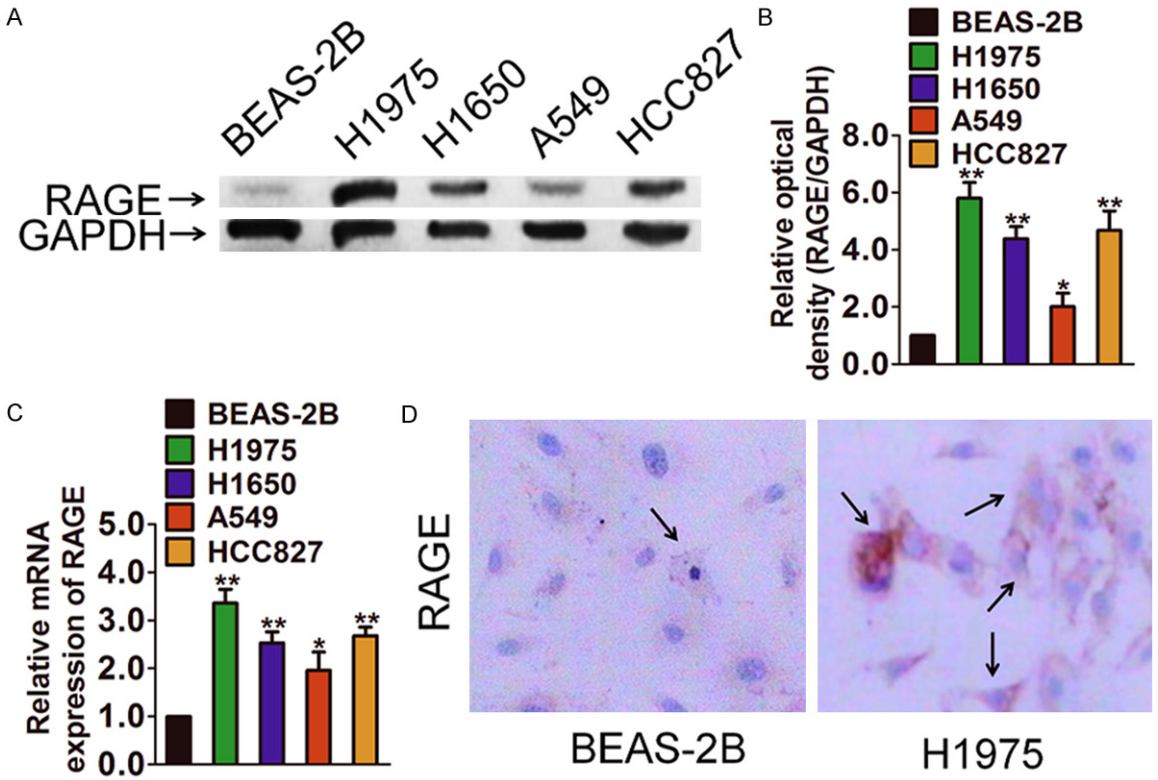


**Figure 2.** RAGE is over-expressed in NSCLC. A. Western blotting analysis of the expression levels of RAGE in various NSLCL cell lines (H1975, A549, H1650 and HCC827) and human bronchial epithelial cells BEAS-2B. B. Expression of RAGE was quantified, normalized to that in BEAS-2B cells. The result was presented from three independent experiments. $*P < 0.05$ and $**P < 0.01$ vs. the BEAS-2B cells. C. qRT-PCR analysis of RAGE mRNA levels in various NSCLC cell lines. PCR values were normalized to the mRNA level of GAPDH. Data was presented as the mean ± SD from three independent measurements. $**P < 0.01$ compared with the BEAS-2B cells. D. Representative immunohistochemical analysis of RAGE (arrows) and cell nucleus (blue) in H1975 and BEAS-2B cells.

## Results

### *RAGE is highly expressed in NSCLC cell lines*

We extracted data on the expression for RAGE from the Oncomine database for esophageal, lung, kidney, and ovarian cancers, focusing on clinical specimens, and compared them to healthy-patient datasets. The Oncomine analysis showed significant levels of RAGE over-expression in lung cancer, but strong RAGE under-expression in other cancer types, including kidney cancer (**Figure 1A**). RAGE levels in human lung cancer tissues were examined with available datasets from the public Oncomine database. As shown in **Figure 1B**, representative results from the Bhattacharjee dataset [23] exhibited a considerably higher level of RAGE expression in lung cancer tissues in contrast to the normal lung tissues ($P < 0.05$). We also tested for the effect of differential RAGE expression on patient free survival by Kaplan-Meier plotter analysis (http://kmplot.com/analysis/). As shown in **Figure 1C**, this analysis showed a correlation between over-expression of RAGE and lower survival rates. Non-small cell lung cancer (NSCLC) represents 80% of the total lung cancer cases and is comprised of adenocarcinoma, adenosquamous carcinoma, squamous cell carcinoma and large cell carcinoma (LCC) subtypes. To investigate any association between RAGE and NSCLC, a western blot was carried out to measure the expression of RAGE in several non-small cell carcinoma cell lines (H1975, A549, H1650 and HCC827). All four cell lines, especially the H1975 cells, had an elevated level of RAGE expression compared to the healthy (non-neoplastic) human bronchial epithelial cells BEAS-2B (**Figure 2A**). The expression of RAGE in the immunoblot assay results was normalized between samples, using a GAPDH control. As shown in **Figure**

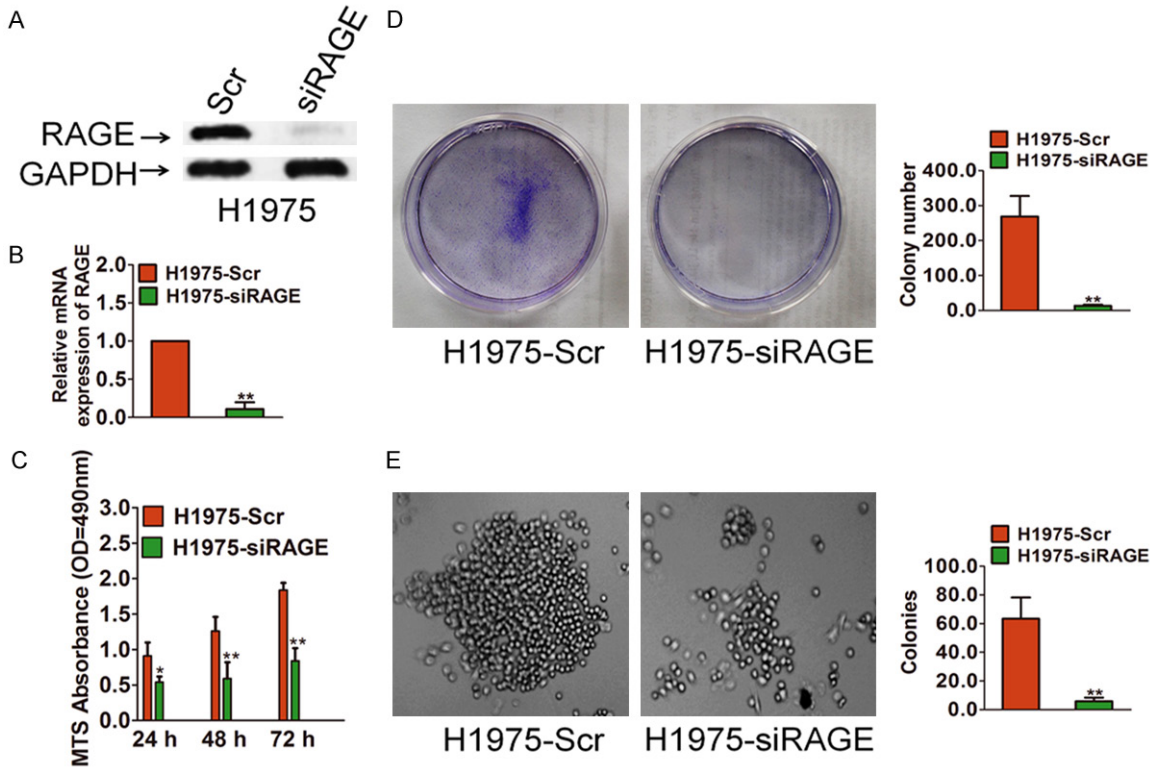


**Figure 3.** Effect of silencing RAGE on H1975 cells proliferation and colony formation. A. H1975 cells were transfected with control siRNA or siRNA against RAGE. After 48 hours of post transfections, RAGE expression determined by western blot in cells. B. H1975 cells were transfected with control siRNA or siRNA against RAGE. RT-PCR analysis indicated effective down-regulation of RAGE after siRNA transfection. The data were indicated as mean ± SD. As indicated by the comparison, $*P < 0.05$ and $**P < 0.01$. C. H1975 cells were transfected with RAGE siRNA for 48 h and completed cells were exposed to complete medium) for 24 hours, 48 hours and 72 hours respectively. The viability of cell was decided using MTS assay kit. The data were indicated as mean ± SD. The data are indicated as mean ± SD (As indicated by comparisons, $*P < 0.05$, $**P < 0.01$). D. The inhibiting of RAGE expression blocked colony forming capabilities of H1975 cells. In 48 hours after transfection, H1975 cells could grow in 1% agar in RPMI-1640 attached with 10% FBS for total 14 days. The representative pictures of H1975 cells anchorage-independent growth were shown. The data were indicated as mean ± SD of triplicates experiments. $**P < 0.01$ compared with the cells transfected with scrambled siRNA. E. Number of multicellular colonies was reduced by RAGE siRNA. Colonies with > 50 cells per colony were counted. The average number of established colonies per field was presented as mean ± SD (n = 5 fields). $**P < 0.01$ versus control.

**2B**, the expression of RAGE was higher in A549 (2.2-fold), H1650 (4.8-fold), HCC827 (5.1-fold), and most significantly in H1975 (5.9-fold), compared to that in the healthy BEAS-2B cells. The NSCLC H1975 cell line is a widely-used model to study the growth and metastasis of lung cancer, characterized by its high metastatic potential and malignancy. We elected to use this cell line for our subsequent experiments based on its elevated RAGE expression. By real-time RT-PCR, we also confirmed that RAGE expression was significantly up-regulated in a variety of human NSCLC cell lines by comparing the expression to that in the healthyBEAS-2B cells (**Figure 2C**). Finally, cell immunohistochemistry demonstrated that RAGE is more highly expressed in H1975 cells relative to that in BEAS-2B cells (**Figure 2D**).

*Silencing RAGE with siRNAs in H1975 cells inhibits growth and colony formation*

To examine the functional role of RAGE in H1975 cells, we knocked down RAGE through the transfection of targeted siRNA. Both protein and mRNA of RAGE were efficiently depleted in transfected H1975 cells (**Figure 3A** and **3B**). Since RAGE has the ability to accelerate the multiplication of tumor cells, we next examined the malignant proliferation of RAGE-silenced H1975 tumor cells. As shown in **Figure 3C**, silencing RAGE reduced the survivability of

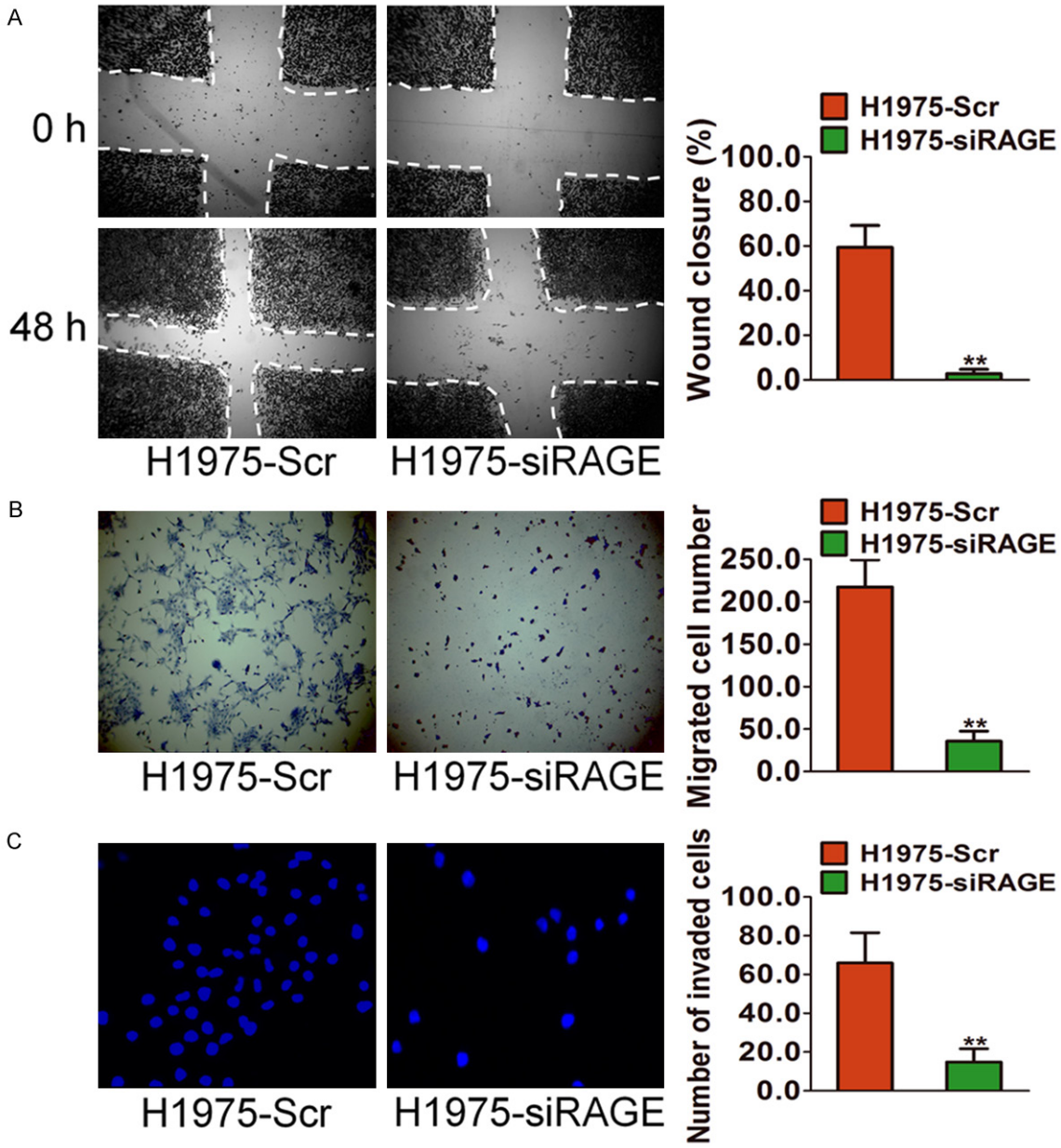


**Figure 4.** RAGE silencing suppresses H1975 cells migration and invasion. A. Wound healing assay was finished to examine the movement of H1975 cells after the silencing of RAGE. Two days later after siRNA was transfected, the cells were supervised at 0 h and 48 h with the focused wound healing capacity. The rate that moves towards the scratched area decided the migration rate, which was determined by the Image J software. B. H1975 cells were decided by un-coated Transwell Boyden chambers determined the migratory abilities of H1975 cells. Post transfection H1975 cells could movement across the bottom of Transwell through pores. The migrated cells were stained with 0.1% crystal violet and counted in five random microscopic field images. C. H1975 cell invasion was examined in the matrigel-coated Transwell chambers. Cells invaded the matrigel-coated insert were stained and fixed with Hochest 33258. Invasive cells were quantitative by counting in five random microscopic field images. All experiments were done in triplicate and the data were indicated as mean ± SD. $^{**}P < 0.01$ in comparison with the control group.

H1975 cells 48 hours post-transfection compared to control H1975 cells transfected with scrambled siRNA. Next, we investigated the effect on anchorage-independent growth, a well-known and important characteristic of cancer cells. Soft agar analysis of H1975 cells was conducted to evaluate whether RAGE knockdown influenced the colony-forming ability of NSCLC cells. Results shown in **Figure 3D** indicate that transiently silencing RAGE dra-

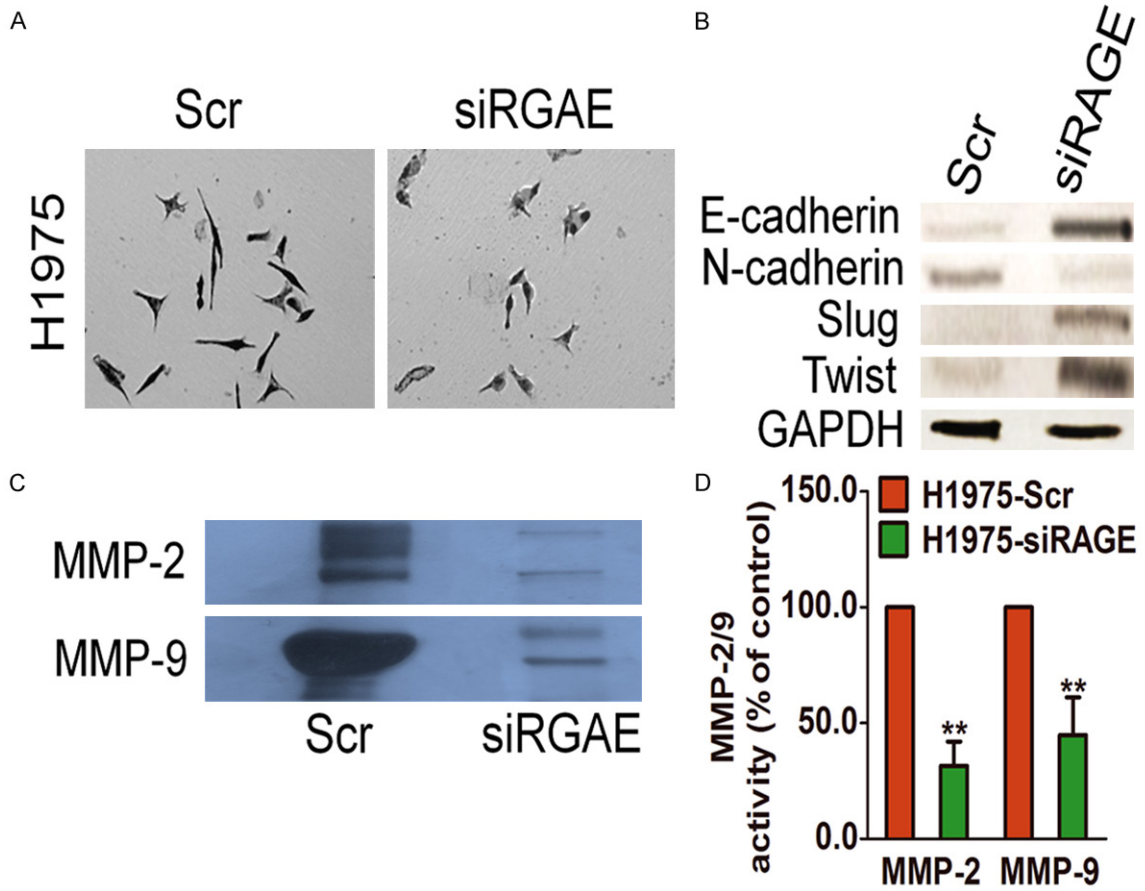


**Figure 5.** RAGE silencing inhibits EMT of H1975 cells. A. H1975 cells were transiently transfected scrambled control or siRAGE, the morphological change of transfected cells was examined 5 days later by microscope. B. RAGE silencing inhibited the expression of several mesenchymal markers and accelerated epithelial marker. Total proteins lysates from RAGE silenced H1975 cells and scrambled control were analyzed for the expression of N-cadherin, E-cadherin, Slug, and Twist with internal control GAPDH. GAPDH functioned as an internal control. C. Gelatin zymography of activated MMP-2 and MMP-9 in the siRNA control-transfected and siRAGE-transfected H1975 cells. D. Activity of MMP-2/9 in control cells and RAGE siRNA cells was determined by MMP-2/9 activity assay. The data were indicated as mean ± SD of triplicates experiments. $*P < 0.05$, $**P < 0.01$ compared with the siRNA control.

matically rescued the colony forming capacity of H1975 cells. We further examined if RAGE siRNA would be sufficient to prevent H1975 cell tumorigenicity. Using an *in vitro* clonogenic assay, we found that down-expressed RAGE was sufficient to significantly reduce the number of tumor colonies of H1975 cells (**Figure 3E**). Taken together, the results indicated a crucial carcinogenicrole for RAGE in the growth of aggressive lung cancer cells.

*RAGE silencing suppressed H1975 cell migration and invasion*

Decreased colony-forming ability is often accompanied by a decreased metastatic potential in tumor cells. The effectiveness of wound healing was used to assess any role for RAGE in regulating the metastatic ability of H1975 cells. An artificial wound was scratched into a monolayer to monitor the migrating progress of both control and RAGE siRNA transfected H1975 cancer cells. As indicated in **Figure 4A**, H1975 cells transfected with RAGE siRNA exhibited reduced migratory capability compared to control cells transfected with scrambled siRNA. Control H1975 cells reformed an integrated monolayer within 48 hours, whereas an integrated monolayer was not reformed within 48 h in RAGE suppressed H1975 cells. Suppressed migration of RAGE silenced H1975 cells was confirmed by a Transwell migration assay. RAGE siRNA-transfected H1975 cells showed consistently less migration across the chamber compared to cells transfected with scrambled

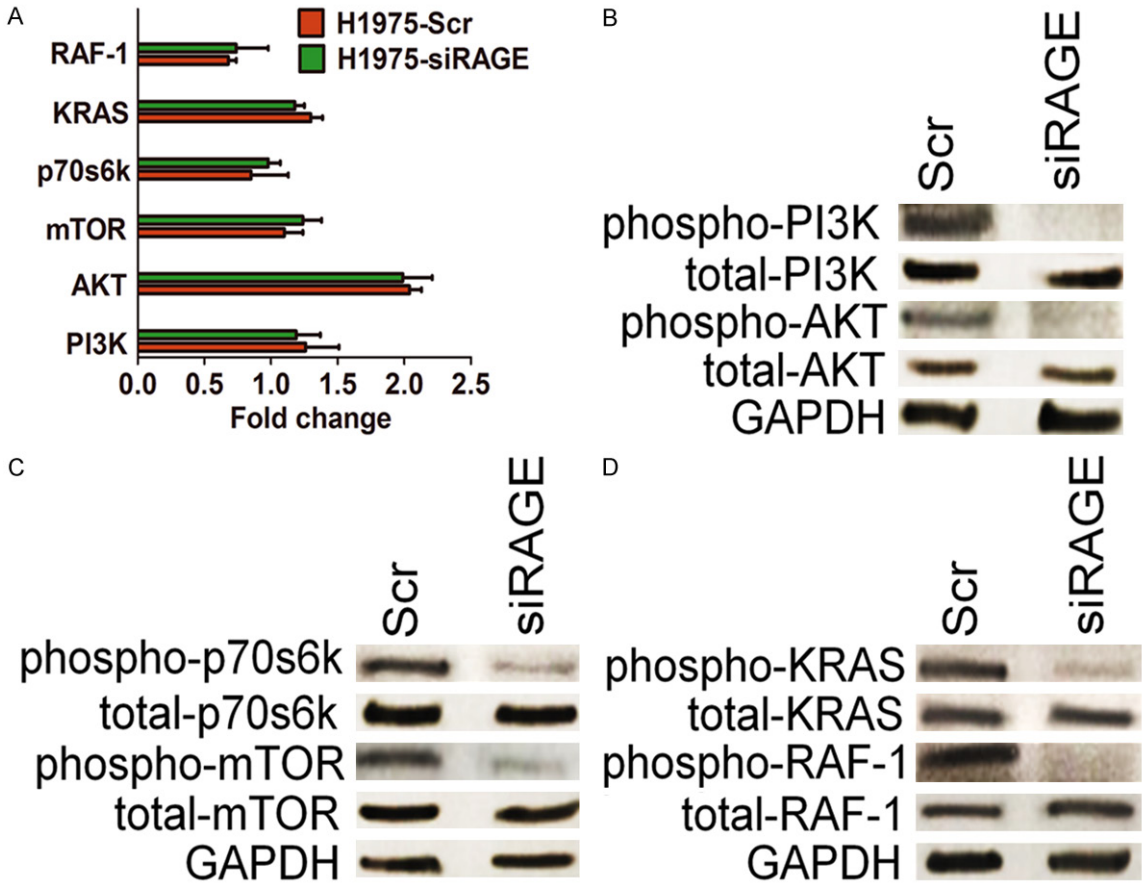


**Figure 6.** Silencing RAGE inhibits PI3K/AKT and KRAS/RAF-1 in H1975 cells. A. qRT-PCR analysis of PI3K, AKT, mTOR, KRAS, RAF-1 and p70s6k mRNA levels in H1975-siRAGE and H1975-Scr cells. PCR values in H1975-siRAGE cells were compared to the levels of H1975-Scr cells. Data was presented as the mean ± SD from three independent measurements. B. H1975 cells were transfected with RAGE siRNA or control siRNA and then were subjected to western blot for measuring protein levels of phosphor-PI3K, t-PI3K, phosphor-AKT and t-AKT respectively. C. H1975 cells were transfected with RAGE siRNA or control siRNA and then were subjected to western blot for measuring protein levels of phospho-p70s6k, t-p70s6k, phosphor-mTOR and t-mTOR respectively. D. The levels of phosphor-KRAS, t-KRAS, phosphor-RAF-1 and t-RAF-1 in indicated H1975 cells were examined by western blotting. GAPDH was used as a loading control.

siRNA, (**Figure 4B**). Cell invasion is one of the most important steps in tumor cells leaving their initial site and eventually entering the lymphatic system and the blood circulation. We performed Matrigel invasion assays and used Transwell invasion chambers to mimic the basement membrane of tumors and estimate the invasion potential of RAGE siRNA-transfected H1975 cells in vitro. RAGE-silenced H1975 cells displayed reduced migratory ability and a major reduction of invasion ability compared to control cells transfected with scrambled siRNA-transfected (**Figure 4C**). In conclusion, these results strongly indicated that blocking RAGE expression suppressed the migration ability and the invasive properties of H1975 NSCLC cells.

*Silencing RAGE blocks EMT in H1975 cancer cells*

Epithelial-mesenchymal transition (EMT) is the first step of cancer cell invasion and the start of the metastatic cascade. Cells which undergo EMT will obtain stem cell-like properties such as increased cell multiplication and metastasis. To find out what role, if any, RAGE may play in the EMT process, the morphological alterations and epithelial or mesenchymal marker changes in RAGE-siRNA H1975 cells were assessed by microscopy. As shown in **Figure 5A**, whereas control H1975 cells maintained cell-cell adhesion, down-expression of RAGE in H1975 cells by siRNA infection damaged cell-cell connections, with cells becoming scattered

and gaining a variable cell shape. Levels of EMT-related factors such as E-cadherin, Twist and Slug were significantly increased upon silencing RAGE in H1975 cells (**Figure 5B**). Notably, western blot analysis of RAGE-suppressed cells showed decreased expression of N-cadherin; loss of this cell-cell adhesion molecule is supposed to promote invasion and metastasis (**Figure 5B**). Matrix metalloproteinases (MMP), which are capable of degrading the various structural components of the ECM, play a critical role in tumor invasion and metastasis. Up-regulation of matrix metalloproteinase (MMP)-2/9 has been considered one of the markers for the metastasis of tumor cells. To determine if reduced RAGE expression affects the activity of MMP-2/9, we tested the effect of RAGE silencing on the activity of MMP-2/9 by gelatin zymography. As expected, both MMP-9 and MMP-2 were significantly down-regulated after RAGE siRNA transfection (**Figure 5C**). Consistent with this result, the activities of MMP-2/9 were reduced inH1975 cells transfected with RAGE siRNA (**Figure 5D**). These data indicate that silencing RAGE in H1975 cells results in the inhibition of proteins involved in EMT and metastasis in NSCLC.

*Silencing RAGE alters the levels of key signaling molecules in H1975 cells metastasis*

To clarify the signaling pathways involved in RAGE-mediated H1975 cell metastasis, we next compared the transcriptional profile of genes involved in metastasis between H1975-siRAGE cells and control cells by real-time PCR. We verified that RAGE coordinates multiple potential regulators of cellular signaling pathways involved in tumor cell multiplication, invasion, migration and EMT. This comprehensive approach revealed that the mRNA levels of several pro-metastasis molecules, including mammalian target of rapamycin (mTOR), KRAS, RAF-1, and protein kinase B (AKT),were not inhibited by siRAGE (**Figure 6A**). Western blot analysis was performed to determine the expression levels of the AKT/PI3K signaling cascade in RAGE siRNA-transfected cells, including downstream and up-stream effectors. As shown in **Figure 6B**, the basal level of AKT activation (p-AKT) was significantly down-regulated in H1975 cells transfected with RAGE siRNA, and the total AKT levels remained stable. Phosphatidylinositol 3-kinase (PI3K) is an important upstream regulator of AKT and its expression was hindered by RAGE suppression compared to that in the control cells transfected with a scrambled siRNA. Activating P13K leads to the phosphorylation of AKT and this triggers the activation of mTOR and the downstream effect or kinase p-p70s6k. Consistently, the active forms of mTOR and p70s6k were inhibited in cells with silenced RAGE (**Figure 6C**). Therefore, we conclude that RAGE silencing hinders the phosphorylation of AKT, PI3K, mTOR, and p-70s6k in H1975 cells and hence hinders cell multiplication and metastasis. KRAS and Raf-1 kinase signaling is one of the most commonly regulated pathways in most cancers, including NSCLC. This pathway may control multiplication, survival, motility, mitosis, apoptosis and differentiation. KRAS and RAF-1 are particularly attractive targets for the development of drugs that could cure cancer. Hence, we investigated the effects of RAGE silencing on the expression of KRAS and RAF-1. As shown in **Figure 6D**, RAGE silencing led to effective reduction of p-KRAS andp-RAF-1with no changes in total protein levels. In summary, our results suggest that the PI3K/AKT and KRAS/RAF-1 pathways act downstream of RAGE and are involved in the metastasis and proliferation of NSCLC.

*Silencing RAGE suppresses growth of tumor in vivo*

Although demonstrated in vitro, we next wanted to confirm that RAGE down-regulation could inhibit tumorigenesis in vivo. For this purpose, we implanted H1975 cells transfected with either RAGE siRNA or scrambled controls in the right flanks of nude mice (n = 3). Mice weights and subcutaneous tumors volumes were analyzed quantitatively once every 3 days. Tumor growth was significantly reduced when RAGE was silenced in H1975 cells, as visually shown in **Figure 7A**. We found that RAGE silencing markedly inhibited weight and tumor volume compared to controls. The average tumor volume in all experimental mice implanted with RAGE-silenced H1975 cells was 472 $mm^3$ (± 175), as compared with 1376 $mm^3$ (± 121) for the control group by the endpoint of the experiment (**Figure 7B**). Accordingly, the tumors induced by the RAGE-siRNA H1975 cells were lighter in weight compared to the scramble control tumors (**Figure 7C**). Consistently, cellular proliferation antigen, Ki67 staining in the tumor

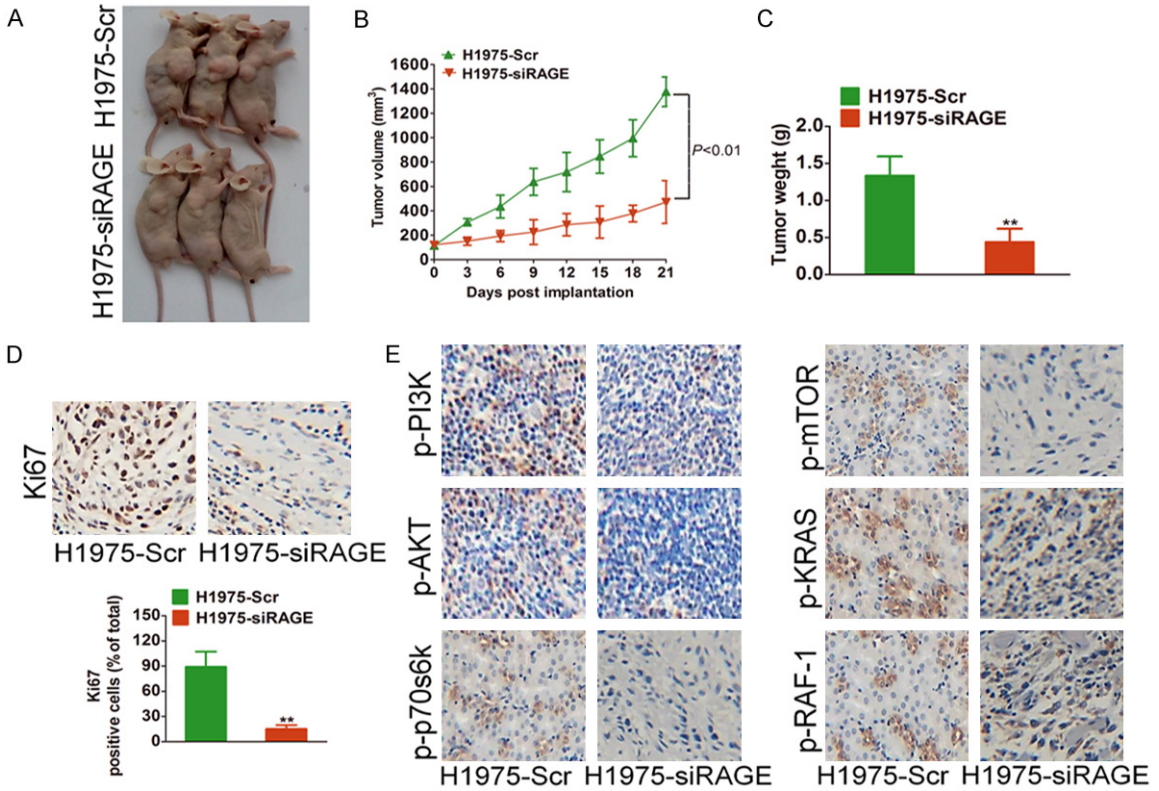


**Figure 7.** Down-regulation of RAGE suppresses H1975 cells growth in vivo. A. Xenografted tumor of H1975 cells transfected with siRNA RAGE or control siRNA in nude mice. B. Quantitative analysis of tumor volumes. The graph shown changes in the volume of tumors. The data are the mean ± SD (n = 3). C. Weights of xenografts established by subcutaneous transplantation with siRNA RAGE-transfected and control-siRNAH1975 cells (n = 3) $^{**}P < 0.01$ compared to the mice inoculated withH1975-Scr cells. D. Representative images of the Ki67-stained tumor sections from mice inoculated with control or siRAGE H1975 cells (upper). Quantification of the number of Ki67 positive cells was shown in the bar graph below. n = 3 in each group. Data in this figure were presented as the mean ± SD. $^{**}P < 0.01$ compared to the mice inoculated with H1975-Scr cells. E. Mice bearing H1975-siRGAE tumor xenograft were sacrificed at the end of the experiment. The tumor tissues were removed for immunohistochemistry analysis with phosphor-KRAS, phosphor-RAF-1, phosphor-AKT, phosphor-PI3K, phospho-p70s6k and phosphor-mTOR antibodies.

tissues from xenografts was markedly inhibited when compared to those from mice inoculated with H1975-Scr (**Figure 7D**). Furthermore, we evaluated the effects of RAGE down-regulation on PI3K/AKT and KRAS/RAF-1 to investigate the mechanism of the tumor growth inhibitory effect induced by RAGE siRNA. As shown in **Figure 7E**, the phosphorylation of PI3K, AKT, KRAS, RAF-1, mTOR, and p70s6k in the siRAGE-treated group were significantly suppressed. These data strongly indicated that RAGE was implicated in NSCLC cell line growth in vivo.

## Discussion

The receptor for advanced glycation end products (RAGE) is a member of the immunoglobulin superfamily of cell surface molecules. It binds many inflammatory ligands, including the S100 family of proteins, amyloid-β peptide, high mobility group box 1 peptide (HMGB-1) and AGE (advanced qlycation end products) [24]. The expression and levels of serum RAGE are dramatically higher in patients with cancer and the gene is implicated in a wide range of malignant tumor types. However, any potential role for RAGE in non-small cell lung cancer (NSCLC) has not been completely elucidated [25]. Our study investigated the potential function of RAGE in NSCLC, demonstrating that RAGE is involved in NSCLC growth and metastasis. We have shown that RAGE expression was increased significantly in NSCLC cell lines and other human non-small cell lung carcinoma tissues. RAGE has been predicted to participate in tumor cell migration and cell invasion

owing to its involvement in the expression of key pro-metastasis molecules. Our experiments show that the migratory and invasive potential of NSCLC cells are significantly attenuated by the knockdown of RAGE via siRNA. In addition, we identified RAGE as a factor involved in epithelial-mesenchymal transition (EMT), with depletion of RAGE leading to a transmutation from mesenchymal to epithelial markers in cell lines. Anchorage-independent growth and decreased proliferation was observed in RAGE-silenced cancer cells, demonstrating that the ability for NSCLC cells to proliferate aberrantly was significantly inhibited. Finally, our in vivo studies indicate that RAGE siRNA inhibited NSCLC tumor growth and proliferation in a mouse model. Our data therefore demonstrates that RAGE may be involved in NSCLC growth and metastasis, consistent with research on other cancers [26].

Tumor metastasis is a contributor to the poor prognosis of patients with NSCLC, with EMT considered a key initial event [27]. During metastasis, polarized epithelial and non-motile cells lose the junctions between cells and turn into individual, motile, non-polarized, and invasive mesenchymal cells. It is possible to control and induce EMT through growth and differentiation factors such as TGF-β, Wnt and Notch, as well as the tyrosine kinase receptor pathway [28]. Migration and invasion are known to be essential parts of cancer cell metastasis to distant organs. In a recent research [29], RAGE was observed to be an important driving force for EMT in a variety of tumor types. Similar to previous results, when we reduced RAGE expression, we observed reduced migratory capability and motility in NSCLC H1975 cells. Reducing expression of RAGE resulted in increased E-cadherin, a hallmark of epithelium-liked tumor cells. Our data also shows that RAGE knockdown leads to the enhancement of other important regulators of EMT, such as Slug and Twist, and decreased the levels of mesenchymal protein N-cadherin. This demonstrates that this is a close relationship between RAGE expression, NSCLC metastasis, and EMT.

The signal transduction cascade downstream of RAGE, such as the PI3K/AKT and KRAS/RAF-1 pathways, was effectively suppressed by RAGE knockdown. Hyper-activated AKT and PI3K have been found in many cancers, including NSCLC, and are involved in increased proliferation, cell growth, metastasis, and angiogenesis [30]. Our data demonstrate that RAGE silencing negatively regulated the PI3K/AKT tyrosine kinase activities. It also affects pathways even further downstream, including mTOR/p70S6K. RAS genes and other components of the RAS/RAF pathway are frequently associated with several hematopoietic malignancies and many other cancers. Notably, the RAGE siRNA reduced the phosphorylation activation of KRAS and RAF-1 simultaneously in NSCLC cells. The levels of mTOR, the signaling effect or molecule of PI3K/AKT, were also significantly suppressed by RAGE silencing and may contribute to the observed decreased proliferation of NSCLC cells. Significantly, RAGE silencing blocked the phosphorylation of the mTOR downstream target p70S6K. Overall, our research proves that silencing RAGE signaling inhibits the downstream PI3K/AKT pathways.

In conclusion, our study shows that the expression of RAGE is up-regulated in NSCLC cells, and silencing RAGE in NSCLC cells inhibits colony-forming ability, proliferation, migration, and the invasive potential. Our data demonstrate that this may be through inhibition of EMT, as key molecular pathways controlling EMT are affected by RAGE silencing in NSCLC cells, included the PI3K/AKT and KRAS/RAF-1 signaling cascades. All this highlights the potential of targeting RAGE as a therapeutic strategy for NSCLC.

## Disclosure of conflict of interest

None.

**Address correspondence to:** Yan Xia Yu, Cancer Treatment Research Center, Qilu Hospital of Shandong University, No.107, Wenhua West Road, Jinan, China. E-mail: yuxiaoyanyuxy@126.com

## References

[1] Paleiron N, Bylicki O, Andre M, Riviere E, Grassin F, Robinet G and Chouaid C. Targeted therapy for localized non-small-cell lung cancer: a review. Onco Targets Ther 2016; 9: 4099-4104.

[2] Cao W, Ribeiro Rde O, Liu D, Saintigny P, Xia R, Xue Y, Lin R, Mao L and Ren H. EZH2 promotes malignant behaviors via cell cycle dysregulation and its mRNA level associates with prognosis of patient with non-small cell lung cancer. PLoS One 2012; 7: e52984.

[3] Boolell V, Alamgeer M, Watkins DN and Ganju V. The evolution of therapies in non-small cell lung cancer. Cancers (Basel) 2015; 7: 1815-1846.
[4] Silvoniemi M, Vasankari T, Loyttyniemi E, Valtonen M and Salminen E. Symptom assessment for patients with non-small cell lung cancer scheduled for chemotherapy. Anticancer Res 2016; 36: 4123-4128.
[5] Wu A, Li J, Wu K, Mo Y, Luo Y, Ye H, Mai Z, Guo K, Wang Y, Li S, Chen H, Luo W and Yang Z. LATS2 as a poor prognostic marker regulates non-small cell lung cancer invasion by modulating MMPs expression. Biomed Pharmacother 2016; 82: 290-297.
[6] Wang X, Cui E, Zeng H, Hua F, Wang B, Mao W and Feng X. RAGE genetic polymorphisms are associated with risk, chemotherapy response and prognosis in patients with advanced NSCLC. PLoS One 2012; 7: e43734.
[7] Nasser MW, Ahirwar DK and Ganju RK. RAGE: a novel target for breast cancer growth and metastasis. Oncoscience 2016; 3: 52-53.
[8] Yue L, Zhang Q, He L, Zhang M, Dong J, Zhao D, Ma H, Pan H and Zheng L. Genetic predisposition of six well-defined polymorphisms in HMGB1/RAGE pathway to breast cancer in a large Han Chinese population. J Cell Mol Med 2016; 20: 1966-1973.
[9] Sakellariou S, Fragkou P, Levidou G, Gargalionis AN, Piperi C, Dalagiorgou G, Adamopoulos C, Saetta A, Agrogiannis G, Theohari I, Sougioultzis S, Tsioli P, Karavokyros I, Tsavaris N, Kostakis ID, Zizi-Serbetzoglou A, Vandoros GP, Patsouris E and Korkolopoulou P. Clinical significance of AGE-RAGE axis in colorectal cancer: associations with glyoxalase-I, adiponectin receptor expression and prognosis. BMC Cancer 2016; 16: 174.
[10] Herwig N, Belter B, Wolf S, Haase-Kohn C and Pietzsch J. Interaction of extracellular S100A4 with RAGE prompts prometastatic activation of A375 melanoma cells. J Cell Mol Med 2016; 20: 825-835.
[11] Qie GQ, Wang CT, Chu YF and Wang R. Expression of HMGB1/RAGE protein in renal carcinoma and its clinical significance. Int J Clin Exp Pathol 2015; 8: 6262-6268.
[12] Bao JM, He MY, Liu YW, Lu YJ, Hong YQ, Luo HH, Ren ZL, Zhao SC and Jiang Y. AGE/RAGE/Akt pathway contributes to prostate cancer cell proliferation by promoting Rb phosphorylation and degradation. Am J Cancer Res 2015; 5: 1741-1750.
[13] Chen JS, Huang XH, Wang Q, Huang JQ, Zhang LJ, Chen XL, Lei J and Cheng ZX. Sonic hedgehog signaling pathway induces cell migration and invasion through focal adhesion kinase/AKT signaling-mediated activation of matrix metalloproteinase (MMP)-2 and MMP-9 in liver cancer. Carcinogenesis 2013; 34: 10-19.
[14] Subramani R, Lopez-Valdez R, Arumugam A, Nandy S, Boopalan T and Lakshmanaswamy R. Targeting insulin-like growth factor 1 receptor inhibits pancreatic cancer growth and metastasis. PLoS One 2014; 9: e97016.
[15] Yuan G, Yan S, Xue H, Zhang P, Sun J and Li G. JSI-124 suppresses invasion and angiogenesis of glioblastoma cells in vitro. PLoS One 2015; 10: e0118894.
[16] Zhang J, Zhu L, Fang J, Ge Z and Li X. LRG1 modulates epithelial-mesenchymal transition and angiogenesis in colorectal cancer via HIF-1alpha activation. J Exp Clin Cancer Res 2016; 35: 29.
[17] Kuang J, Li L, Guo L, Su Y, Wang Y, Xu Y, Wang X, Meng S, Lei L, Xu L and Shao G. RNF8 promotes epithelial-mesenchymal transition of breast cancer cells. J Exp Clin Cancer Res 2016; 35: 88.
[18] Wang L, Shi Y, Ju P, Liu R, Yeo SP, Xia Y, Owlanj H and Feng Z. Silencing of diphthamide synthesis 3 (Dph3) reduces metastasis of murine melanoma. PLoS One 2012; 7: e49988.
[19] Li Y, Shang P, Qian AR, Wang L, Yang Y and Chen ZN. Inhibitory effects of antisense RNA of HAb18G/CD147 on invasion of hepatocellular carcinoma cells in vitro. World J Gastroenterol 2003; 9: 2174-2177.
[20] Khotskaya YB, Goverdhan A, Shen J, Ponz-Sarvise M, Chang SS, Hsu MC, Wei Y, Xia W, Yu D and Hung MC. S6K1 promotes invasiveness of breast cancer cells in a model of metastasis of triple-negative breast cancer. Am J Transl Res 2014; 6: 361-376.
[21] Hong L, Pan F, Jiang H, Zhang L, Liu Y, Cai C, Hua C, Luo X, Sun J and Chen Z. miR-125b inhibited epithelial-mesenchymal transition of triple-negative breast cancer by targeting MAP2K7. Onco Targets Ther 2016; 9: 2639-2648.
[22] Xu Y, Lin H, Meng N, Lu W, Li G, Han Y, Dai X, Xia Y, Song X, Yang S, Wei Y, Yu L and Zhao Y. YL529, a novel, orally available multikinase inhibitor, potently inhibits angiogenesis and tumour growth in preclinical models. Br J Pharmacol 2013; 169: 1766-1780.
[23] Bhattacharjee A, Richards WG, Staunton J, Li C, Monti S, Vasa P, Ladd C, Beheshti J, Bueno R, Gillette M, Loda M, Weber G, Mark EJ, Lander ES, Wong W, Johnson BE, Golub TR, Sugarbaker DJ and Meyerson M. Classification of human lung carcinomas by mRNA expression profiling reveals distinct adenocarcinoma subclasses. Proc Natl Acad Sci U S A 2001; 98: 13790-13795.
[24] Wang H, Li Y, Yu W, Ma L, Ji X and Xiao W. Expression of the receptor for advanced glyca-

tion end-products and frequency of polymorphism in lung cancer. Oncol Lett 2015; 10: 51-60.
[25] Basappa, Rangappa KS and Sugahara K. Roles of glycosaminoglycans and glycanmimetics in tumor progression and metastasis. Glycoconj J 2014; 31: 461-467.
[26] Hermani A, De Servi B, Medunjanin S, Tessier PA and Mayer D. S100A8 and S100A9 activate MAP kinase and NF-kappaB signaling pathways and trigger translocation of RAGE in human prostate cancer cells. Exp Cell Res 2006; 312: 184-197.
[27] Gawlik-Rzemieniewska N, Galilejczyk A, Krawczyk M and Bednarek I. Silencing expression of the NANOG gene and changes in migration and metastasis of urinary bladder cancer cells. Arch Med Sci 2016; 12: 889-897.
[28] Chiang KC, Hsu SY, Lin SJ, Yeh CN, Pang JH, Wang SY, Hsu JT, Yeh TS, Chen LW, Kuo SF, Cheng YC and Juang HH. PTEN insufficiency increases breast cancer cell metastasis in vitro and in vivo in a xenograft zebrafish model. Anticancer Res 2016; 36: 3997-4005.
[29] Nasser MW, Wani NA, Ahirwar DK, Powell CA, Ravi J, Elbaz M, Zhao H, Padilla L, Zhang X, Shilo K, Ostrowski M, Shapiro C, Carson WE 3rd and Ganju RK. RAGE mediates S100A7-induced breast cancer growth and metastasis by modulating the tumor microenvironment. Cancer Res 2015; 75: 974-985.
[30] Moore J, Megaly M, MacNeil AJ, Klentrou P and Tsiani E. Rosemary extract reduces Akt/mTOR/p70S6K activation and inhibits proliferation and survival of A549 human lung cancer cells. Biomed Pharmacother 2016; 83: 725-732.