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ORIGINAL RESEARCH

RETRACTED ARTICLE: Matrine suppresses the migration and invasion of NSCLC cells by inhibiting PAX2-induced epithelial-mesenchymal transition

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Abstract: Non-small cell lung cancer (NSCLC) is the major cause of deaths among all the cancer types worldwide. Most of the NSCLC is diagnosed at an advanced stage and the 5-year overall survival rate is low. The reason for the low survival rate of patients with NSCLC is mainly due to distant metastasis. Matrine, a traditional Chinese medicine, has been shown a significant anti-proliferation and anti-invasive effect in tumors. However, little is known on the anti-invasive mechanism of matrine in lung cancer. Therefore, we tried to investigate the molecular mechanism of matrine on the invasive ability of NSCLC cells in vitro. Cell Counting Kit-8 assay was used to evaluate the cell viability. Transwell assay was used to detect the migration and invasion abilities. Microarray assay was used to analyze the differentiated expression genes with or without matrine treatment. Western blotting and real-time polymerase chain reaction were applied to detect the expressions of *PAX2*, E-cadherin and N-cadherin. Our study showed that matrine could suppress the proliferative activity of NSCLC cells in a dose- and time-dependent manner. Further investigation discovered that the migration and invasion of NSCLC cells were significantly inhibited by treatment with different concentrations of matrine. Microarray assay, real-time polymerase chain reaction and western blotting showed that matrine could significantly decrease the expression of PAX2. In addition, epithelial-mesenchymal transition and related proteins were decreased. In conclusion, matrine may block PAX2 expression to interfere with epithelial-mesenchymal transition signaling pathway that ultimately inhibit the migration and invasion of NSCLC cells in vitro. Matrine might serve as a potential agent for NSCLC treatment.

Keywords: matrine, PAX2, epithelial-mesenchymal transition, migration, invasion

Introduction

Lung cancer is the major cause of deaths among all the cancer types. Lung cancer is divided into 2 broad histological subtypes: small cell lung cancer (accounting for 15% of lung cancers) and non-small cell lung cancer (NSCLC, nearly 85% of all lung cancers).[1–3] Most of the lung cancer is diagnosed at an advanced stage and the 5-year overall survival rate is <5%.[4] One of the main reasons for the low survival rate of lung cancer patients is metastasis.[5] Metastatic lung cancer is responsible for >90% of lung cancer-related deaths.[6,7] Therefore, inhibition the metastasis of lung cancer might decrease the incidence of mortality and improve the overall survival.

Matrine, a compound extracted from *Sophora flavescens* Ait, has been used for viral hepatitis, arrhythmia and skin inflammation.[8] Recently, the anti-tumor activities of matrine were reported. Some studies have shown that matrine had suppression effect on nasopharyngeal carcinoma,[9] NSCLCs,[10] breast cancer,[11] hepatocellular cancer,[12]

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cholangiocarcinoma cells,[13] colorectal cancer cells[14] and acute myeloid leukemia cells.[15] Furthermore, matrine was also reported to reduce the migratory and invasive abilities of tumor cells,[9,16–19] including lung cancer cells.[20,21] However, further studies are needed to clarify the molecular mechanisms by which matrine inhibits the migration and invasion of lung cancer cells.

In the present study, microarray assay was performed to reveal the underlying mechanism by which matrine inhibited the migration and invasion ability of NSCLC cells. Our data found that *PAX2*, a migration-related gene, was significantly down-regulated by matrine treatment. Further investigation showed that matrine reduced the epithelial-mesenchymal transition (EMT) of lung cancer cells. We speculated that matrine might suppress the migration and invasion of NSCLCs by inhibiting EMT via PAX2. For the first time, we found that PAX2 was a potent target of matrine, which might be a therapeutic target of lung cancer.

Materials and methods

Cell lines and agents

The A549 and H1299 human NSCLC cells were kindly gifted by Prof Ranyi Liu (Cancer Center of Sun Yat-sen University, Guangzhou, China) and cultured in Roswell Park Memorial Institute-1640 (Gibco, Thermo Fisher Scientific Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific Inc.), 100 U/mL penicillin and 100 µg/mL streptomycin on culture plates at 37°C in a 5% CO_2 atmosphere with constant humidity. Matrine was purchased from DiBo Biological Science and Technology Limited Liability Company (Shanghai, China). Matrine powder was formulated with a ddH_2O concentration of 50 mg/mL solution, and placed in 4°C storage. This research was reviewed and approved by the Institutional Research Ethics Committee of Affiliated Hospital of Guangdong Medical University.

Cytotoxicity assay

Cell proliferation was detected with Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Tokyo, Japan). A549 or H1299 cells were plated in flat-bottomed 96-well microplates at a density of 2,000/well. After seeding, the new medium contained different concentrations of matrine (0, 0.25, 0.5, 1, 2, 4 and 8 mg/mL) cultured for 24, 48 and 72 hours. Cells were further incubated for specified time and measured with CCK-8 at a concentration of 10% for 2 hours, and absorbance values were collected in an automated microplate reader (Thermo Electron Corp., Shanghai, China) at 450 nm. Cell viability rate was calculated as per the following equation: viability rate (%) = ([T − B)/(U − B]) ×100%, where T is the treated cell absorbance, U is the untreated cell absorbance and B is the background absorbance when neither drug nor CCK-8 was added. All experiments were repeated at least 3 times independently.

Migration and invasion assays

Transwell chamber with 8 µm filter inserts (BD Biosciences, San Jose, CA, USA) without matrigel was used to measure cell migration potential. For invasion assay, 50 µL of matrigel (BD Biosciences) was coated at the bottom of the upper chamber, according to the manufacturer's guidelines. Cells (5×10^4) were added to the upper chamber with different concentration matrine (0, 0.5 and 2 mg/mL) without serum. The lower chamber was filled with 600 µL medium with 10% FBS. After 24 hours of incubation, cells in the upper surface of the insert membrane were wiped thoroughly with a cotton swab to remove any non-migrated or non-invasive cells. The outside of inserts was fixed with methanol for 20 minutes, and then stained with 0.1% crystal violet for 20 minutes. The migration and invasion cells were digitally photographed using an optical microscope in a magnified 200× field of view (EVOS XL Core; Life Technologies, Madison, WI, USA). The cells in 5 randomly fields were photographed and subjected to cell counting. The number of cells in the 5 fields of view was averaged. Experiment was repeated at least 3 times and submitted by the mean ± SD.

Microarray analysis

The total RNA was extracted from the A549 cell treated with 0.5 mg/mL matrine for 24 hours using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions, and each sample was performed in triplicate. Cell was then frozen at −80°C and a gene chip assay was performed by Shanghai Biotechnology Company (SBC). The gene expression profile was performed by SBC using the AffymetrixPrimeView™ Human Gene Expression Array (Affymetrix, Inc., Santa Clara, CA, USA). In strict accordance with the instructions to carry out all the data analysis, and the SBC online system serviced by the company (http://www.shbiochip.bioon.com.cn/) was also used for data analysis. The original data of the file was normalized by Robust Multi-array Average background correction, and the values were converted by log2. The different genes were selected according to fold change ≥2.0. Change in gene expression profile of the heat map was mapped with HemI 1.0 software.

Real-time polymerase chain reaction

Total RNA from A549 and H1299 cells were extracted with TRIzol (Invitrogen) and reversely transcribed to first-strand complementary DNA with the PrimeScript™ RT-PCR kit (Takara, Shiga, Japan). Primers of *PAX2*, matrix metalloproteinase (MMP)2, MMP9, E-cadherin, N-cadherin and the internal standard glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were synthesized by Sangon Biotechnology Co., Ltd (Shanghai, China). Reactions were amplified in a Prism 7500 RT-PCR machine (Applied Biosystems, Foster City, CA, USA) carried out under the following conditions: initial denaturation at 95°C for 10 minutes, 35 cycles of denaturation at 95°C for 20 seconds, annealing at 60°C for 10 seconds, and polymerization at 72°C for 30 seconds. Gene specific primers are listed in Table 1.

Western blotting analysis

Cells were plated into 6 cm dishes, treated with or without matrine at the concentration as indicated (0, 0.5, 1 and 2 mg/mL) for 24 hours. After washed with PBS for 3 times, the cells were collected and lysed with 150–200 μL of lysis buffer (20 mM Tris [pH7.5], 150 mM NaCl, 1% Triton X-100, sodium pyrophosphate, β-glycerophosphate, EDTA, Na_3VO_4 and sleupeptin) containing 1/100 phenylmethanesulfonyl fluoride solution. Protein in loading buffer was denatured by heating at 100°C for 10 minutes. The lysates were electrophoretically separated by 10% or 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred into the polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). Membrane blocked in defatted milk (BD Biosciences) was dissolved in 5% Tris-buffered saline with Tween-20 buffer (TBST) at room temperature for 1 hour. The blots were then incubated with various primary antibodies overnight at 4°C, and then incubated with corresponding goat anti-rabbit (A0208, 1:5,000; Beyotime Institute of Biotechnology, Shanghai, China) or anti-mouse (A0216, 1:5,000; Beyotime Institute of Biotechnology) IgG-horseradish peroxidase-conjugated secondary antibody for 1 hour at room temperature. After that, the membrane was washed with TBST 3 times for 10 minutes each time. The signals were visualized with ECL Plus substrates (Applygen Technologies Inc., Beijing, China). The details of the used primary antibodies are as follows: PAX2 (PB0776; Boster Biological Technology, Wuhan, China), N-cadherin (D4R1H; Cell Signaling Technology, Boston, MA, USA), E-cadherin (24E10; Cell Signaling Technology), MMP2 (4022; Cell Signaling Technology), MMP9 (3852; Cell Signaling Technology), GAPDH (AG019; Beyotime Biotech). All experiments were performed for 3 times at least.

Table 1 Specific primers used in polymerase chain reaction amplification

Gene sequence	
PAX2	Forward 5′-AAGCACTTGCGAGCTGACAC-3′
	Reverse 5′-ATGTGCTCTGATGCCTGGAA-3′
N-cadherin	Forward 5′-GCAATTGATGCTGACGATCC-3′
	Reverse 5′-CCAGCTGCCACTGTGATGAT-3′
E-cadherin	Forward 5′-TGAGGCCAAGCAGCAGTACA-3′
	Reverse 5′-GGCTTCATTCACATCCAGCA-3′
MMP2	Forward 5′-CCAAGTCTGGAGCGATGTGA-3′
	Reverse 5′-GGAGTCCGTCCTTACCGTCA-3′
MMP9	Forward 5′-TCATCTTCCAAGGCCAATCC-3′
	Reverse 5′-GCAGAAGCCGAAGAGCTTGT-3′
GAPDH	Forward 5′-TGACTTCAACAGCGACACCCA-3′
	Reverse 5′-CACCCTGTTGCTGTAGCCAAA-3′

Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MMP, matrix metalloproteinase.

Statistical analysis

Data were analyzed using SPSS 13.0 (SPSS, Inc., Chicago, IL, USA). Continuous data are expressed as the mean ± SD. For 2-group comparison, the Student's *t*-test method was used. For more than 2-group comparison, 1-way analysis of variance was used to assess statistical significance. Values of $P<0.05$ were considered statistically significant.

Results

Matrine inhibits the proliferation of A549 and H1299 cells

CCK-8 assay was used to detect the effect of matrine on the viability of NSCLC cells. Our results showed that matrine could suppress the proliferative activity of A549 and H1299 cells in a dose- and time-dependent manner (Figure 1). The IC_{50} values of matrine were 2.29±0.40, 1.28±0.11 and 0.65±0.06 mg/mL in A549 cell and 1.35±0.25, 0.83±0.23 and 0.59±0.18 mg/mL in H1299 cell for 24, 48 and 72 hours, respectively. In addition, it appeared that the anti-proliferative effect was not obvious when treated with matrine below the concentration of 0.5 mg/mL, while inhibition of cell growth was remarkable at concentrations of 4 and 8 mg/mL compared with the control groups. These results indicated that matrine could effectively inhibit the proliferation of NSCLC cells.

Matrine suppresses the migration and invasion of NSCLC cells

Our research showed that the migration of NSCLC cell lines was significantly inhibited with different concentrations

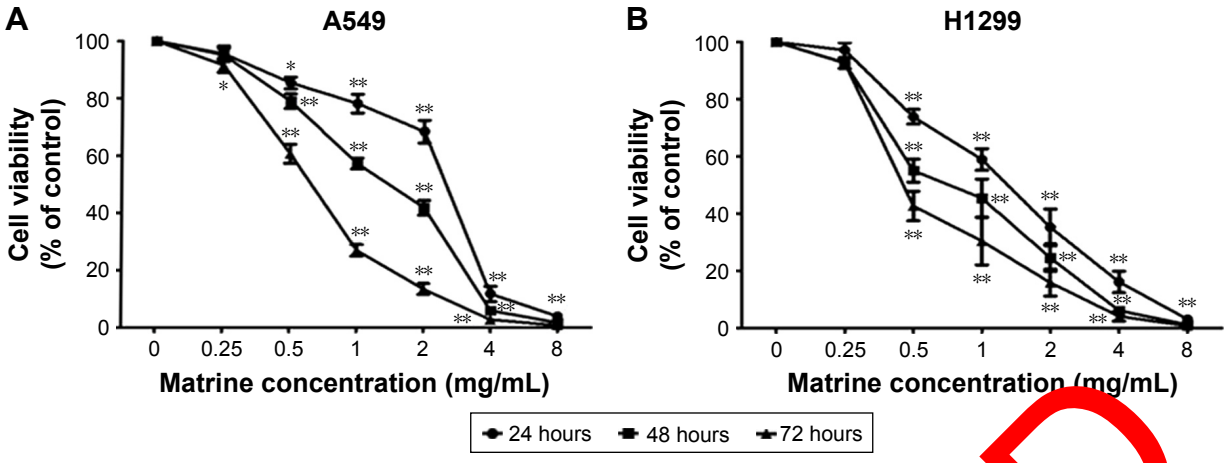

Figure 1 Matrine inhibited the proliferation of A549 and H1299 cells.
Notes: After being treated by matrine with different concentrations (0, 0.25, 0.5, 1, 2, 4 and 8 mg/mL) cultured for 24, 48 and 72 hours, the viability of A549 (**A**) and H1299 (**B**) cells was detected by CCK-8 assay. All data are presented as the mean values ± SD from three independent experiments. *$p<0.05$ and **$p<0.01$ vs control group.
Abbreviation: CCK-8, cell counting kit-8.

of matrine compared with the control group in both A549 (Figure 2A, $p<0.01$) and H1299 (Figure 2B, $p<0.05$) cells. In addition, we analyzed the invasive potential of A549 (Figure 2A, $p<0.05$) and H1299 (Figure 2B, $p<0.05$) cells with or without matrine treatment. As expected, the mean numbers of invasion cells with different concentrations of matrine were significantly lower than that of the untreated group. Therefore, these data indicated that matrine could significantly suppress the migratory and invasive ability of NSCLC cells.

Microarray analyses of gene expression files in matrine-treated A549 cells

To investigate the molecular signaling pathways by which matrine reduces the invasive ability of NSCLC cells, microarray analysis was performed using 3 biological replicates A549 cell treated with or without matrine to determine differential expression genes. Interestingly, 2,419 differentially expressed genes (4.14%) of the total 58,341 probe-sets of A549 cell were identified with a cut-off threshold of 2-fold change (Figure 3A). Approximately 36 genes associated with EMT and migration and invasion were selected from differentially expressed genes for mapping heat maps (Figure 3B). In these 36 genes, *PAX2*, a gene involved in carcinogenesis and migration, was obviously down-regulated. However, the role of PAX2 in the migration and invasion of lung cancer remains unknown. Consequently, we concentrated on PAX2 in the following study.

To confirm the effect of matrine on the expression of PAX2, RT-PCR and western blot analyses were performed to detect the expression of PAX2 mRNA and protein levels, respectively. Interestingly, both the mRNA (Figure 4A) and protein expression levels (Figure 4B) were remarkably decreased with increasing matrine concentration in both A549 and H1299 cells. These results clearly showed that matrine could suppress the expression of PAX2 at both mRNA and protein levels.

Matrine blocks EMT signaling pathway markers

EMT process is a key step of cancer cell invasion and metastasis.[22–24] Previous studies have shown that PAX2 could promote the invasion and metastasis of tumor cells.[25–28] Furthermore, PAX2 was associated with EMT. It was reported that PAX2 was closely related with nephrogenesis and renal cell carcinoma.[29–31] In our study, RT-PCR and western blot analysis were performed to explore the effect of PAX2 on the expression of EMT-related markers in NSCLC cells. In both A549 (Figure 5A) and H1299 (Figure 5B) cells, treatment with different concentrations of matrine resulted in up-regulated E-cadherin mRNA level and down-regulated N-cadherin mRNA level. Consistently, matrine significantly increased the E-cadherin protein level and decreased the N-cadherin protein level. Furthermore, matrine also significantly reduced the expression of MMP2 and MMP9 mRNA and protein level in A549 and H1299 cells. Consequently, we speculated that matrine might suppress the migration and invasion of NSCLCs by inhibiting EMT via PAX2.

Discussion

The present study showed that matrine could inhibit the proliferation, migration and invasion of lung cancer cells (Figures 1 and 2), which is consistent with the previous studies.[9,11,14,16–19,32,33] Several signaling pathways were reported to be involved in the inhibitory impact of matrine on the

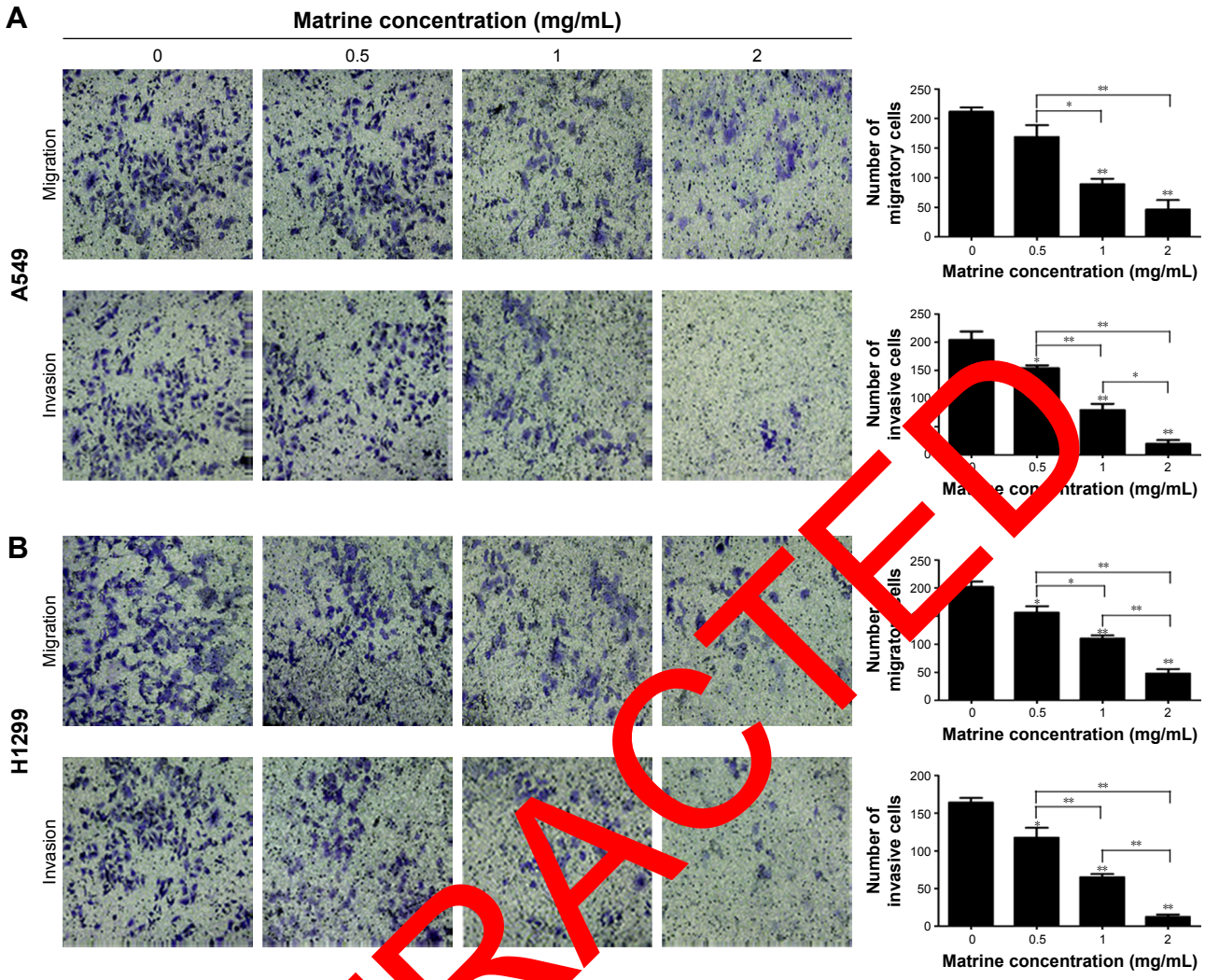

Figure 2 The migration and invasion ability of NSCLC cells were attenuated with the treatment of matrine in a dose-dependent manner in A549 (**A**) and H1299 (**B**) cells.
Notes: For migration assay, cells were treated with different concentrations (0, 0.5, 1 and 2 mg/mL) of matrine following which the cell was transferred to a Transwell chambers without matrigel, and the numbers of cells migration were counted 24 hours later. For invasion assay, cells were pretreated with different concentrations of matrine for 24 hours cultured in matrigel invasion chambers, and the numbers of cells invasion were counted 24 hours later. The numbers of migration and invasion cells were counted and shown as the mean values ± SD from three independent experiments. *$p<0.05$ and **$p<0.01$ vs control group. Magnification: 200×.
Abbreviation: NSCLC, non-small cell lung cancer.

migration and invasion of tumor cells, including nuclear factor-kappa B,[9,14] PI3K/Akt,[15] uPA,[16] p38[17] and Wnt signaling pathways.[19] Signaling pathways involved in migration and invasion have also been reported in matrine-treated lung cancer cells. Liao et al showed that matrine inhibited the migration and invasion of lung cancer cells via elevating the expression of miR-133a to suppress activation of epidermal growth factor receptor/AKT/MMP-9 pathway.[20] Wu et al have indicated that the matrine derivative YF-18 could restore E-cadherin expression and inhibit migration.[35]

To further explore the molecular signaling pathways involved in the anti-invasive effect of matrine, we performed microarray assay to identify the differentiated expression genes after matrine treatment. Interestingly, 2,419 differentially expressed genes were detected (Figure 3). A host of genes were annotated with the migration and invasion. Among them, we focused on PAX2 since PAX2 expression is known to be altered during the EMT process[36] and has been associated with tumor cell migration and invasion.[25–28]

Interestingly, further investigation demonstrated that matrine could inhibit the expression of PAX2 both at the mRNA and protein level, especially at a high concentration (Figure 4). This is the first scientific report, to the best of our knowledge, that matrine can reduce the expression of PAX2. These results indicate that matrine may inhibit the migration and invasion of NSCLC cells by down-regulating the expression of PAX2. *PAX2* is a member of the *PAX* gene family, there are currently 9 family members described and divided into 4 groups according to their structure,[37] which play an important role in the development and spread of a variety of

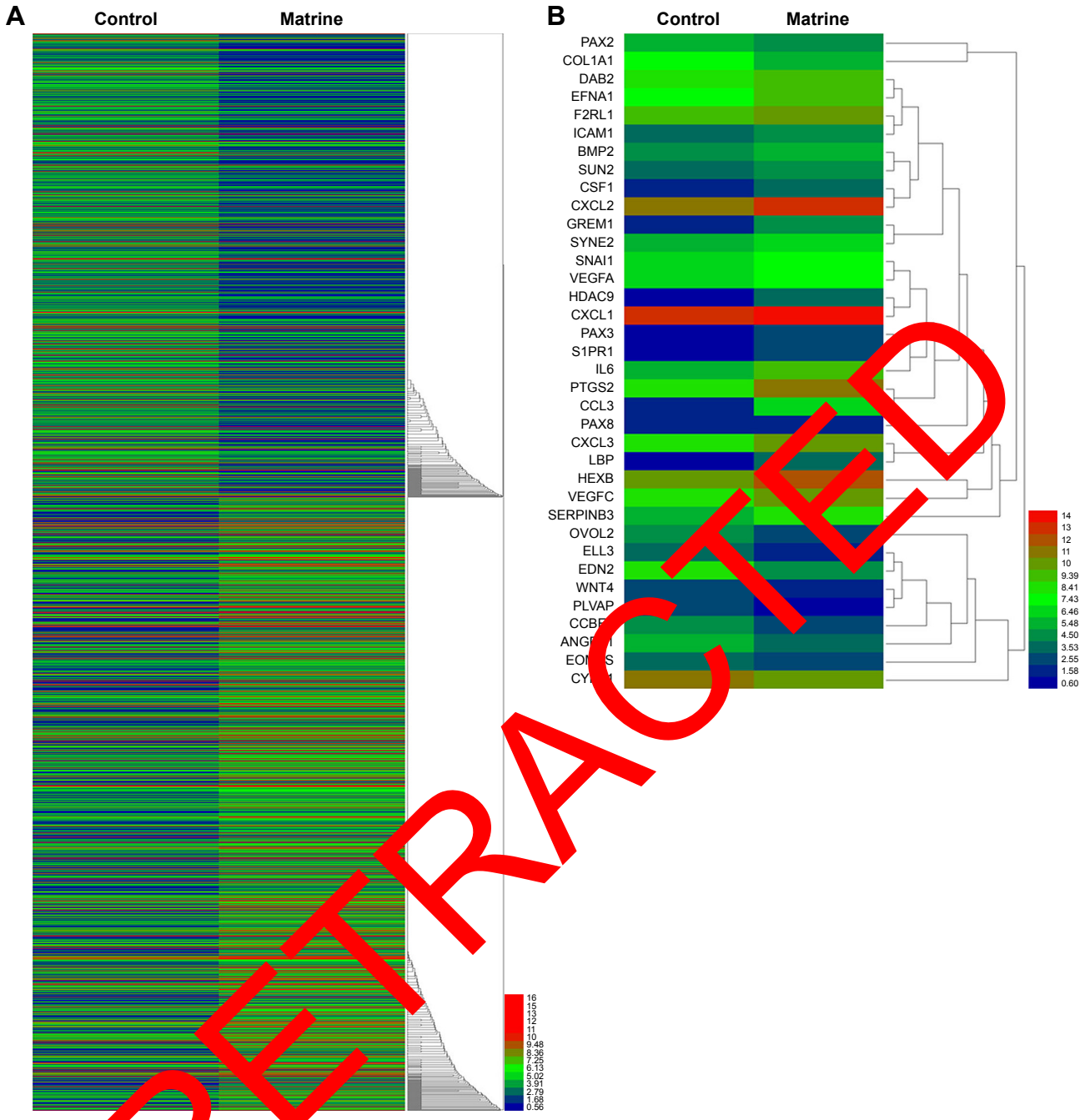

Figure 3 Heat [illegible] analysis [illegible] data in A549 cell treated with or without matrine.
Notes: (**A**) About [illegible] differentially expressed genes with a cut-off threshold of 2-fold were analyzed and shown. (**B**) Approximately 36 genes associated with epithelial-mesenchymal transition, migration and invasion were selected from the differential express genes for mapping heat map. The expression levels of genes were expressed in different colors, red representing increase and blue representing decrease.

cell lines, as well as in the development of organs, tissues, and central nervous system. Moreover, *PAX* genes have been found to be expressed in a variety of cancers and may contribute to tumorigenesis and may also be a useful tumor marker.[38] Recently, PAX2 was also found to be a good marker for renal tumors.[30] In addition, overexpression of PAX2 was closely related to tumor migration and invasion. Takashi Ueda et al have found that PAX2 could promote the invasion of prostate cancer cell by hepotocyte growth factor pathway, and down-regulating *PAX2* might be a successful therapeutic strategy to treat or prevent prostate cancer metastasis.[25,26] The PAX2 expression level was significantly increased in esophageal squamous cell carcinoma (ESCC) tissues and that its expression correlated with the stage, lymph node metastasis and lymphatic invasion of ESCC, and *PAX2* over-expression resulted in markedly increased metastasis capacity

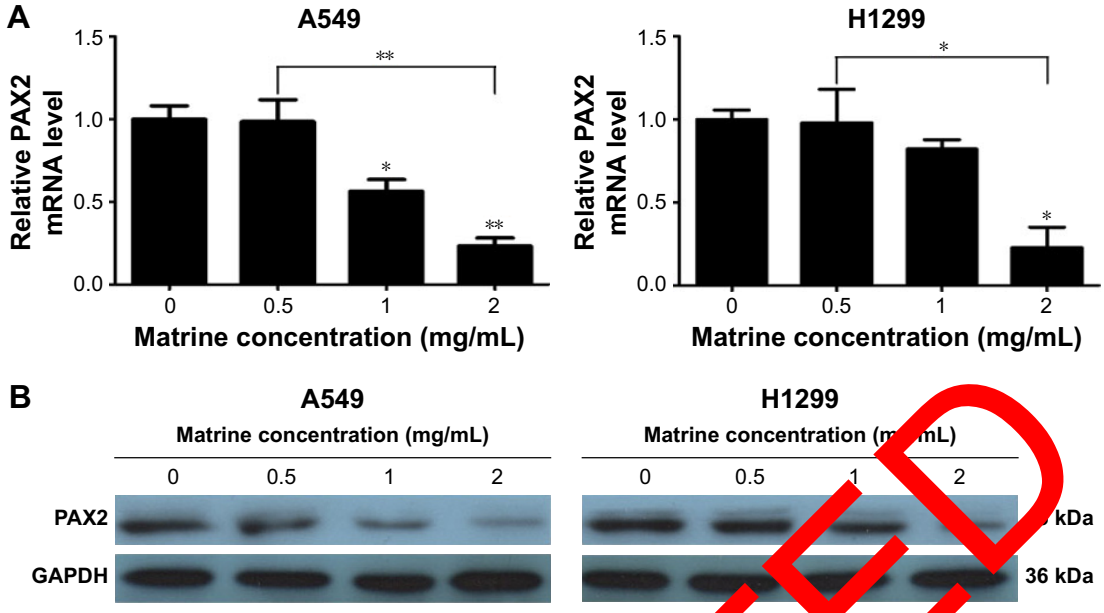

Figure 4 Matrine inhibited the expression of PAX2 in NSCLC cells.
Notes: (**A**) The PAX2 mRNA levels of PAX2 in A549 and H1299 cells were detected by real-time polymerase chain reaction after treatment with different concentrations (0, 0.5, 1 and 2 mg/mL) of matrine for 24 hours. (**B**) The PAX2 protein levels in A549 and H1299 cells were detected by western blot analysis after treatment with different concentrations of matrine for 24 hours. Data are presented as the mean ± SD of 3 independent experiments. *$p<0.05$ and **$p<0.01$ vs control group.
Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; NSCLC, non-small cell lung cancer.

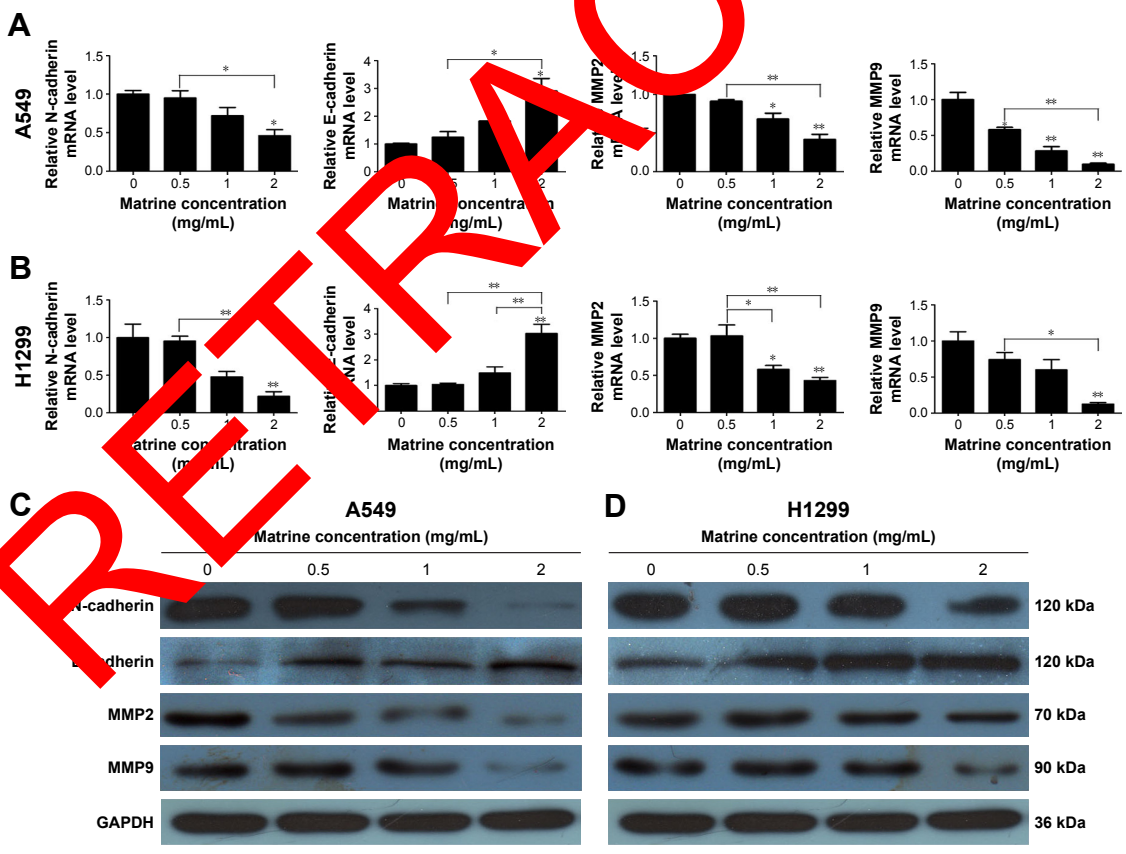

Figure 5 The effects of matrine on the expression of EMT and migration associated molecules.
Notes: The mRNA expression levels of N-cadherin, E-cadherin, MMP2, MMP9 in A549 (**A**) and H1299 (**B**) cells were detected by real-time polymerase chain reaction after treatment with different concentrations of matrine as indicated (0, 0.5, 1, 2 mg/mL). The protein expression levels of N-cadherin, E-cadherin, MMP2, MMP9 in A549 (**C**) and H1299 (**D**) cells were detected by western blotting after treatment with different concentrations of matrine as indicated (0, 0.5, 1, 2 mg/mL). Data are presented as the mean ± SD for mRNA expression levels of A549 and H1299 cells of three independent experiments. *$p<0.05$ and **$p<0.01$ vs control group.
Abbreviations: EMT, epithelial-mesenchymal transition; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MMP, matrix metalloproteinase.

in esophageal cancer cells.[27] Moreover, overexpression of miR-497 in SKOV3 cells induced PAX2 protein expression and resulted in inhibition of cell proliferation, migration and invasion, and induction of cell apoptosis.[39] Therefore, we can infer that matrine might suppress the migration and invasion abilities of NSCLC cells by down-regulation of PAX2.

EMT is a key step in cancer cells migration and invasion.[23,40,41] EMT is a biological process for polarized epithelial cells that lose their cell polarity and adhesion between cells and then acquire mesenchymal cells phenotype. EMT has a complex biological behavior,[42] 2 major changes occur: 1) epithelial cells lose cell–cell adhesion, this course can be confirmed by E-cadherin; 2) mesenchymal components increased, which can be testified by N-cadherin. On the basis of these 2 variations, the cytoskeletal is rearranged and the ability to migration and invasion is enhanced.[41] Moreover, E-cadherin and N-cadherin are essential in regulating the migration and invasion of cancer cells.[43,44] Therefore, these 2 main members of EMT process, E-cadherin and N-cadherin, were detected. Notably, the mRNA and protein expression levels of N-cadherin were down-regulated when treated with increased concentrations of matrine, while the mRNA and protein level of E-cadherin were up-regulated (Figure 5). Regarding the regulating role of PAX2 on EM[illegible] we concluded that matrine might suppress the migration an[illegible] invasion abilities of NSCLC cells by inhibiting EMT via down-regulation of PAX2.

Conclusion

Our data found that *PAX2*, a mi[illegible]n-related g[illegible]e, was significantly down-regulated by matri[illegible] treatment. Further investigation showed [illegible]at matrine mig[illegible] suppress the migration and invasion [illegible] NSCL[illegible]s by inhibiting EMT via PAX2. For the first time[illegible]ve f[illegible]nd that PAX2 was a potent target of matrin[illegible]ich m[illegible]ht be [illegible]herapeutic target of lung cancer.

Acknowle[illegible]gments

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Disclosure

The authors report no conflicts of interest in this work.

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