Glutathione S-transferase ω 1 promotes the proliferation, migration and invasion, and inhibits the apoptosis of non-small cell lung cancer cells, via the JAK/STAT3 signaling pathway

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Abstract. Glutathione S-transferase ω 1 (GSTO1) expression levels have been discovered to be upregulated in various types of cancer. However, to the best of our knowledge, the role of GSTO1 in non-small cell lung cancer (NSCLC) has not been investigated. The present study aimed to investigate the role of GSTO1 in NSCLC and to determine the potential molecular mechanism. GSTO1 expression levels in A549 cells were knocked down using short hairpin RNA and GSTO1 overexpression in H2122 cells was achieved using cDNA constructs. Reverse transcription-quantitative PCR was used to analyze the mRNA expression levels of GSTO1. Cell proliferation was determined using a Cell Counting Kit-8 assay, whereas cell migration and invasion were analyzed using Transwell assays. Flow cytometric analysis was performed to determine the levels of cell apoptosis. The expression levels of GSTO1, Bax, caspase 3, JAK and STAT3 were analyzed using western blotting. The results revealed that GSTO1 overexpression significantly promoted the proliferation, migration and invasion, and inhibited the apoptosis of H2122 cells, whereas the opposite trend was achieved in A549 cells with GSTO1 knockdown. GSTO1 overexpression also significantly increased the phosphorylation levels of JAK and STAT3, whereas the knockdown of GSTO1 promoted the opposite effects. In conclusion, the findings of the present study indicated that GSTO1 may serve as an oncogene in NSCLC. The results suggested that GSTO1 may have an important role in NSCLC by regulating the JAK/STAT3 signaling pathway. Therefore, inhibiting the expression levels of GSTO1 may represent a potential novel therapeutic strategy for NSCLC.

Introduction

Lung cancer is the leading cause of cancer-related mortality worldwide, accounting for an estimated 1.6 million deaths each year (1,2). Non-small cell lung cancer (NSCLC) is the predominant subtype of lung cancer, accounting for 85% of all cases, of which lung squamous cell carcinoma and lung adenocarcinoma are the most common histopathological subtypes (3,4). Although significant progress has been made in the development of treatment strategies in previous years, including surgery, radiotherapy, chemotherapy and targeted treatment, the prognosis of NSCLC remains unsatisfactory, and the 5-year overall survival rate is only 16% for all stages (5,6). Thus, elucidating the molecular mechanisms underlying NSCLC development and progression is crucial for improving the treatment of NSCLC.

Glutathione S-transferases (GSTs) are enzymes that can combine with glutathione and various endogenous and exogenous metabolites during biotransformation (7). In humans, seven cytoplasmic GST classes have been identified, including α, μ, σ, π, τ, ζ and ω (8). GST ω 1 (GSTO1), a member of the ω class of GSTs, has been reported to be associated with several types of cancer. For example, a previous study revealed that polymorphisms in GSTO1 increased the risk of hepatocellular carcinoma (9). In addition, accumulating evidence has reported the relationship between GSTO1 gene polymorphisms and NSCLC (10-12). For instance, Bulus *et al* (13) demonstrated that GSTO1 expression levels in colon cancer cells were significantly upregulated compared with in normal colon epithelial cells. Chuang *et al* (14) illustrated that the expression levels of GSTO1 were upregulated in human bladder cancer cells. Moreover, GSTO1 has been revealed to contribute to cell growth, death, migration and invasion in several types of cancer cell (7,15-17). For example, Wang *et al* (15) reported that GSTO1 was upregulated in cutaneous malignant melanoma (CMM) tissues and cells, where it contributed to CMM cell growth, migration and invasion. Piaggi *et al* (17) identified

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that GSTO1 overexpression was associated with protection against cisplatin-induced apoptosis. However, it remains unclear whether GSTO1 may be involved in the pathogenesis of NSCLC. Thus, the aim of present study was to investigate the role of GSTO1 in NSCLC and to determine the potential molecular mechanism.

Materials and methods

Cell culture. NSCLC cell lines (A549, H2122, H292, H1299 and H460) and normal human lung epithelial cells (BEAS-2B) were purchased from The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences. All cells were maintained in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 μg/ml streptomycin in an incubator at 37°C with a 5% CO_2 atmosphere. Cell lines in the logarithmic growth phase were selected for subsequent experiments.

Cell transfection. Cell transfections were performed using Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Untransfected cells were used as a blank control. Briefly, 4×10^5 H2122 or A549 cells were plated into a six-well plate and cultured to 90-95% confluence. The overexpression of GSTO1 in NSCLC cells was induced through transfection with a pcDNA3.1 plasmid (4 μg; Invitrogen; Thermo Fisher Scientific, Inc.) carrying the GSTO1 cDNA insert, with an empty vector (4 μg) as the negative control (pcDNA3.1-NC). The knockdown of GSTO1 in NSCLC cells was induced using 2 μg short hairpin RNA (shRNA; Thermo Fisher Scientific, Inc.) against GSTO1, using stable non-specific shRNA (2 μg) as the NC (shRNA-NC). The shRNA-NC sequence was 5'-CCGGCCTA AGGTTAAGTCGCCCTCGCTCGAGCGAGGGCGACTTA ACCTTAGGTTTTTC-3' and the shRNA-GSTO1 sequence was 5'-GCTGGAAGCAATGAAGTTA-3'. Cells were transfected for 24 h at 37°C in an atmosphere containing 5% CO_2 to obtain stably transfected cells for future use. At 48 h post-transfection, the overexpression and knockdown of GSTO1 was confirmed using reverse transcription-quantitative PCR (RT-qPCR) and western blotting.

RT-qPCR. Total RNA was extracted from cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Total RNA was reverse transcribed into cDNA using a RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.), which was conducted according to the manufacturer's protocol. qPCR analysis was subsequently performed using a SYBR Green RT-PCR kit (Takara Bio, Inc.) on an ABI 7500 Fast Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The following thermocycling conditions were used for qPCR: Initial denaturation for 10 min at 95°C; followed by 40 cycles of denaturation at 95°C for 15 sec and annealing/extension at 55°C for 45 sec. The following primer sequences were used for the qPCR: GSTO1 forward, 5'-AGCATACCCAGGGAAGAAG-3' and reverse, 5'-CTGCCATCCACAGTTTCAG-3'; and β-actin forward, 5'-CATCGTCCACCGCAAATGCTTC-3' and reverse, 5'-AAC CGACTGCTGTCACCTTCAC-3'. Expression levels

were quantified using the $2^{-\Delta\Delta Cq}$ method (18). β-actin was used as the endogenous control and expression levels were normalized to β-actin.

Western blotting. Total protein was extracted from cells using RIPA lysis buffer (Beyotime Institute of Biotechnology). Total protein was quantified using a Bradford assay and 25 μg total protein/lane was separated via SDS-PAGE on 10% gels. The separated proteins were subsequently transferred onto PVDF membranes (EMD Millipore) and blocked with 5% non-fat milk in TBS with 0.05% Tween-20 for 30 min at room temperature. The membranes were then incubated with the following primary antibodies at 4°C overnight: Anti-GSTO1 (1:500; cat. no. ab129106; Abcam), anti-Bax (1:500; cat. no. ab182733; Abcam), anti-caspase 3 (1:500; cat. no. ab44976; Abcam), anti-JAK (1:500; cat. no. ab47435; Abcam), anti-phosphorylated (p)-JAK (1:500; cat. no. ab138005; Abcam), anti-STAT3 (1:500; cat.no.ab119352;Abcam),anti-p-STAT3(1:500;cat.no.ab30647; Abcam) and anti-β-actin (1:500; cat. no. ab8227; Abcam). Following the primary antibody incubation, the membranes were incubated with anti-rabbit HRP-conjugated secondary IgG antibody (1:50,000; cat. no. ab205718; Abcam) or anti-mouse HRP-conjugated secondary IgG antibody (1:5,000; cat. no. ab205719; Abcam) for 1 h at room temperature. Protein bands were visualized using enhanced chemiluminescence (Pierce; Thermo Fisher Scientific, Inc.). Several X-ray films were analyzed to verify the linear range of the chemiluminescence signals and densitometric analysis was performed using ImageJ software (version 1.41; National Institutes of Health).

Cell Counting Kit-8 (CKK-8) assay. Cell proliferation was analyzed using CCK-8 solution (Beyotime Institute of Biotechnology), according to the manufacturer's protocol. Briefly, cells (1×10^4-10^5 cells/well) were seeded into a 96-well plate with the culture medium and incubated in a CO_2 incubator at 37°C for 24 h. Subsequently, 10 μl CCK-8 solution was added to each well and incubated for another 2 h at 37°C. Finally, the absorbance was measured at 450 nm using a microplate reader.

Cell migration and invasion assays. Cell migration and invasion were analyzed using Transwell plates. For the migration assay, 1.0×10^5 cells in 200 μl serum-free RPMI-1640 medium were plated into the upper chambers of 8-μm Transwell plates. For the invasion assays, 1.0×10^5 cells were plated into the upper chamber of Transwell plates precoated at 37°C for 4-5 h with Matrigel. The lower chambers for both assays were filled with RPMI-1640 medium supplemented with 20% FBS. After 24 h of incubation at 37°C, non-invasive or non-migratory cells were removed from the upper chambers, and invasive or migratory cells in the lower chamber were fixed with 100% methanol for 30 min at room temperature. Finally, cells were stained with 0.1% crystal violet for 30 min at room temperature, and then visualized and counted using an inverted fluorescence microscope (Olympus Corporation; magnification, x100) with ImageJ software (version 1.8; National Institutes of Health).

Flow cytometric analysis of apoptosis. Cell apoptosis was analyzed using an Annexin V-FITC/propidium iodide (PI) apoptosis kit (BD Biosciences). Briefly, 1×10^5 cells were stained
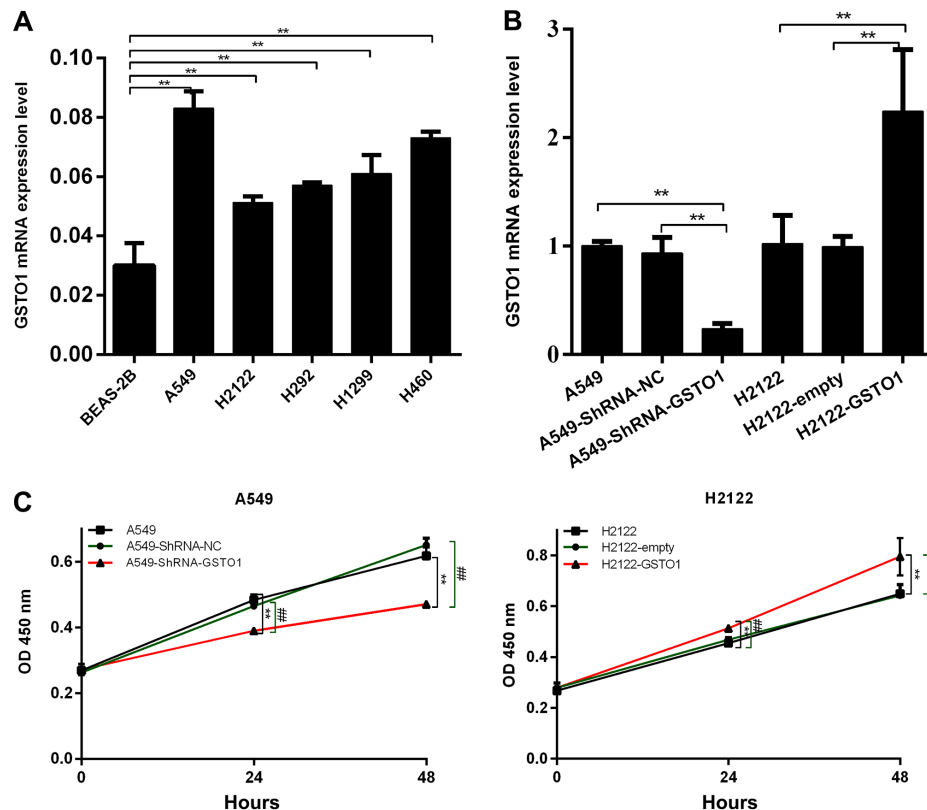
Figure 1. GSTO1 promotes the proliferation of NSCLC cells. (A) RT-qPCR analysis of GSTO1 expression levels in different NSCLC cell lines compared with the BEAS-2B cell line. (B) Expression levels of GSTO1 in NSCLC cells transfected with shRNA-NC, shRNA-GSTO1, pcDNA3.1-empty or pcDNA3.1-GSTO1 were determined using RT-qPCR. (C) Cell proliferation was measured using a Cell Counting Kit-8 assay in NSCLC cells transfected with shRNA-GSTO1 or pcDNA3.1-GSTO1 compared with their respective NCs. Data are presented as the mean ± SD. **P<0.01; ##P<0.01. NSCLC, non-small cell lung cancer; NC, negative control; GSTO1, glutathione S-transferase ω 1; shRNA, short hairpin RNA; RT-qPCR, reverse transcription-quantitative PCR; OD, optical density.

with Annexin V-FITC and PI, according to the manufacturer's protocol. Apoptotic cells were subsequently analyzed with a BD FACSAria™ Fusion flow cytometer (BD Biosciences) using ModFit software version 3.2 (BD Biosciences). The apoptotic rate was calculated as the percentage of early and late apoptotic cells.

Statistical analysis. All statistical analyses were performed using SPSS version 22.0 software (IBM Corp.). Data from each experiment are presented as the mean ± SD of three independent experiments. Statistical differences between ≥3 groups were determined by one-way ANOVA followed by a Tukey's post hoc test for multiple comparisons. P<0.05 was considered to indicate a statistically significant difference.

Results

GSTO1 promotes the proliferation of NSCLC cells. To determine the function of GSTO1 in NSCLC cell lines, GSTO1 expression levels in several NSCLC cell lines were analyzed (Fig. 1A). As the expression levels of GSTO1 were the lowest in the H2122 cell line compared with BEAS-2B cells (P<0.01), these cells were selected to construct GSTO1-overexpressing cells. GSTO1 expression levels were knocked down using shRNA in the A549 cell line, as this cell line expressed the highest levels of GSTO1 compared with BEAS-2B cells (P<0.01). RT-qPCR was subsequently used to confirm the

transfection efficiency through analyzing GSTO1 expression levels in A549 and H2122 cells following the transfections. As shown in Fig. 1B, GSTO1 expression levels were significantly downregulated in A549 cells transfected with shRNA-GSTO1 compared with those in the shRNA-NC and control groups (P<0.01), whereas GSTO1 expression levels were significantly upregulated in H2122 cells transfected with pcDNA3.1-GSTO1 compared with pcDNA3.1-empty and H2122 control cells (P<0.01).

CCK-8 assays were used to investigate the effect of GSTO1 on the proliferation of NSCLC cells. As shown in Fig. 1C, the proliferative ability of A549 cells transfected with shRNA-GSTO1 was significantly inhibited compared with cells transfected with shRNA-NC (P<0.01). Conversely, the proliferative ability was significantly increased in cells transfected with pcDNA3.1-GSTO1 compared with pcDNA3.1-empty- transfected cells (P<0.01). These results indicated that GSTO1 may promote the proliferation of NSCLC cells.

GSTO1 promotes the migration and invasion of NSCLC cells. Transwell assays were used to analyze the effect of GSTO1 on the migratory and invasive abilities of NSCLC cells. The knockdown of GSTO1 in A549 cells by shRNA significantly decreased the cell migratory and invasive abilities compared with the shRNA-NC and control groups (P<0.01), whereas overexpression of GSTO1 with pcDNA3.1-GSTO1
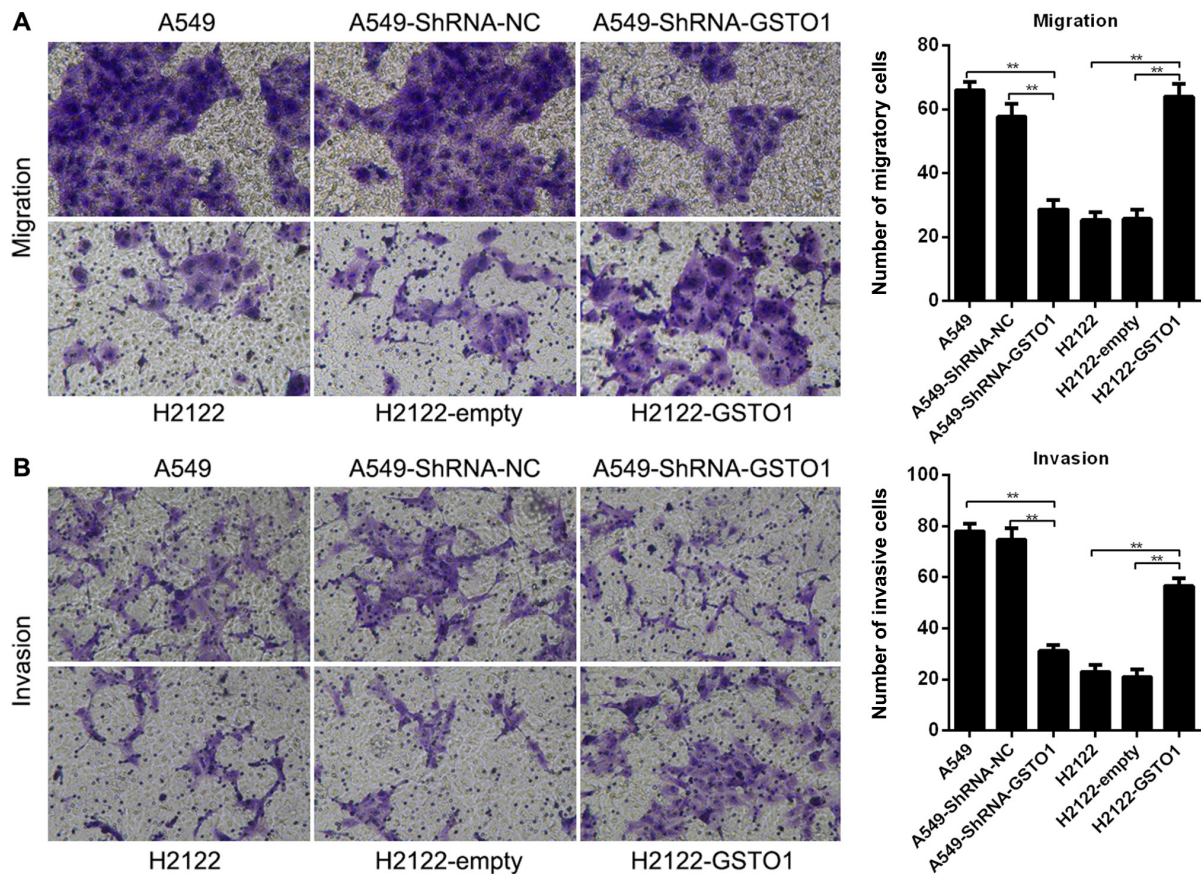
Figure 2. GSTO1 promotes the migration and invasion of NSCLC cells. Cell (A) migration and (B) invasion were detected using Transwell assays in NSCLC cells transfected with shRNA-GSTO1 or pcDNA3.1-GSTO1 compared with their respective NCs (magnification, x200). Data are presented as the mean ± SD. **P<0.01. NSCLC, non-small cell lung cancer; NC, negative control; shRNA, short hairpin RNA; GSTO1, glutathione S-transferase ω 1.

significantly increased the migratory and invasive abilities of H2122 cells compared with the pcDNA3.1-empty-transfected and control cells (P<0.01) (Fig. 2A and B). These results indicated that GSTO1 may promote the migration and invasiveness of NSCLC cells.

GSTO1 inhibits the apoptosis of NSCLC cells. To further confirm the role of GSTO1 in NSCLC cells, the effects of GSTO1 knockdown or overexpression on apoptosis were analyzed using flow cytometry. The knockdown of GSTO1 with shRNA significantly promoted the apoptosis of A549 cells compared with the shRNA-NC and control groups (P<0.01; Fig. 3A). Following successful transfection with pcDNA3.1-GSTO1, the overexpression of GSTO1 was discovered to significantly inhibit the apoptosis of H2122 cells compared with the pcDNA3.1-empty and control groups (P<0.01). Meanwhile, the genetic knockdown of GSTO1 significantly upregulated the expression levels of Bax and caspase 3 compared with the shRNA-NC and control groups (P<0.01), whereas the overexpression of GSTO1 significantly downregulated the expression levels of Bax and caspase 3 compared with the pcDNA3.1-empty and control groups (P<0.01) (Fig. 3B). These results indicated that GSTO1 may inhibit the apoptosis of NSCLC cells.

GSTO1 activates the phosphorylation of JAK and STAT3. To determine the possible mechanisms underlying the

GSTO1-mediated increases in the aggressive phenotypes observed in NSCLC cells, the effects of GSTO1 on JAK and STAT3 expression levels were analyzed. The results revealed that the genetic knockdown of GSTO1 in A549 cells significantly decreased the phosphorylation levels of JAK and STAT3 compared with the shRNA-NC and control groups (P<0.01), whereas pcDNA3.1-GSTO1-transfected H2122 cells had significantly increased phosphorylation levels of JAK and STAT3 compared with pcDNA3.1-empty-transfected and control cells (P<0.01) (Fig. 3B). These results indicated that GSTO1 may promote aggressive phenotypes in NSCLC cells via activation of the JAK/STAT3 signaling pathway.

Discussion

Despite recent advances in the treatment of NSCLC, the long-term survival rate of NSCLC remains low, with a 5-year overall survival rate of 16% (5,6,19,20). Therefore, further investigations into the mechanism underlying NSCLC development and progression are essential for improving the treatment of NSCLC. The expression levels of GSTO1 were previously discovered to be upregulated in various types of cancer, including lymphoma, melanoma and colorectal cancer (7,16,21). However, to the best of our knowledge, the role of GSTO1 in NSCLC has not been investigated. The present study aimed to investigate the role of GSTO1 in NSCLC and to determine the potential molecular mechanism.
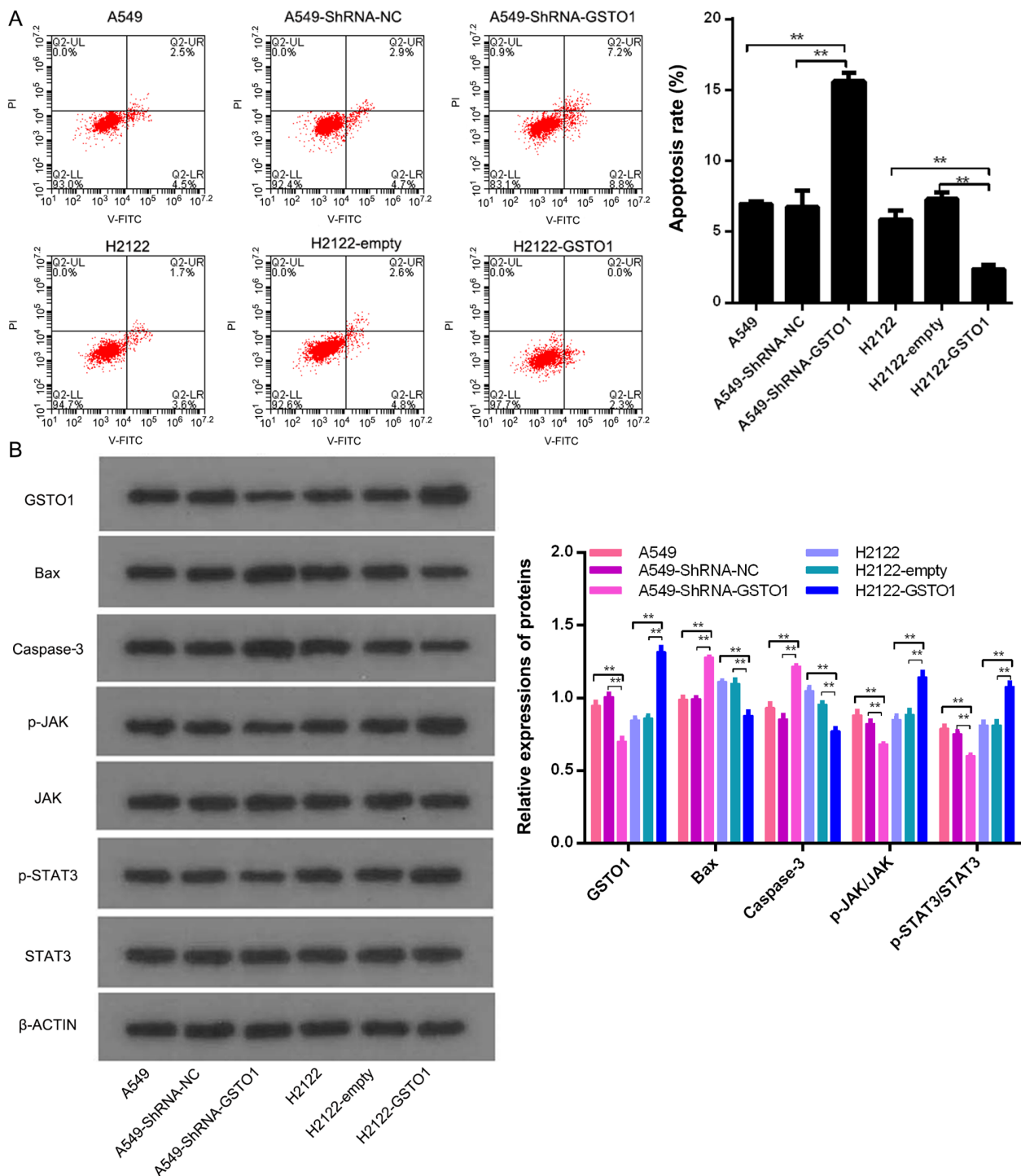
Figure 3. GSTO1 inhibits the apoptosis of NSCLC cells. (A) Cell apoptosis was analyzed using flow cytometry in NSCLC cells transfected with shRNA-GSTO1 or pcDNA3.1-GSTO1 compared with their respective NCs. (B) Protein expression levels of apoptosis-related proteins (Bax and caspase 3), p-JAK and p-STAT3 were analyzed using western blotting. Data are presented as the mean ± SD. **P<0.01. NSCLC, non-small cell lung cancer; NC, negative control; shRNA, short hairpin RNA; GSTO1, glutathione S-transferase ω 1; p-, phosphorylated.

To determine the relationship between GSTO1 and NSCLC, a series of experiments was performed to investigate the effect of GSTO1 on NSCLC *in vitro*. The current study first verified the function of GSTO1 in NSCLC cell lines. The results demonstrated that GSTO1 overexpression significantly promoted the proliferation, migration and invasion, and inhibited the apoptosis of NSCLC cells, whereas knockdown of GSTO1 exerted the opposite effects. Similarly, Wang *et al* (15)

reported that silencing GSTO1 could inhibit the growth and aggressiveness of CMM cells, promote cell cycle arrest and increase cell apoptosis. In addition, Piaggi *et al* (17) revealed that GSTO1 overexpression was associated with the protection against cisplatin-induced apoptosis. These results indicated that GSTO1 may serve as an oncogene in NSCLC.

To further investigate the possible mechanisms of the GSTO1-mediated increase in the aggressive phenotypes

observed in NSCLC cells, the effects of GSTO1 on JAK and STAT3 were investigated. STAT3 is an oncogene, which is known to promote the proliferation, motility, progression and survival of cancer cells (22). STAT3 is mainly located in the cytoplasm, where it can be phosphorylated by JAK-mediated tyrosine phosphorylation following the stimulation by cytokines (23). p-STAT3 subsequently translocates into the nucleus where it acts as transcription factors for numerous genes involved in cellular apoptosis and proliferation (24,25). Persistent activation of the JAK/STAT3 signaling pathway has been observed in various types of cancer, including NSCLC (26). In the present study, GSTO1 overexpression was revealed to significantly increase the phosphorylation levels of JAK and STAT3. This finding indicated that GSTO1 may promote aggressive phenotypes in NSCLC cells via activation of the JAK/STAT3 signaling pathway. Future research should aim to determine the possible mechanism of the GSTO1-mediated increase in aggressive phenotypes through Gene Ontology functional term and Kyoto Encyclopedia of Genes and Genomes signaling pathway enrichment analyses.

In conclusion, the findings of the present study indicated that GSTO1 may promote the proliferation, migration and invasion, and inhibit the apoptosis of NSCLC cells via promoting the phosphorylation of JAK and STAT3. These findings may provide crucial molecular insights into NSCLC pathogenesis and further provide a theoretical basis for NSCLC treatment.

Acknowledgements

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

KW and WJ conceived and designed the study; KW and FLZ performed the experiments; KW analyzed and interpreted the data; KW drafted the manuscript; and WJ provided administrative support. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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