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SLC12A8 promotes the migration and invasion of non-small cell lung cancer (NSCLC) cells

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Abstract. This study aims to investigate the impacts of SLC12A8 on the invasion, migration, and epithelial-mesenchymal transition (EMT) of non-small cell lung cancer (NSCLC) cells. GEPIA database was employed to examine SLC12A8 expression pattern in lung cancer cells. Subsequently, qRT-PCR and Western blot analyses were conducted to assess SLC12A8 expression in NSCLC tissues and cell lines. The overall prognosis of NSCLC patients was evaluated using Kaplan-Meier plot and univariate and multivariate COX regression curves. The knockdown of SLC12A8 was established using lentivirus-mediated shRNA in A549 and H1299 cells. Cell proliferation, invasion, migration, and apoptosis were evaluated using CCK-8 assay, transwell, and flow cytometry techniques, respectively. Western blot analysis was performed to measure the expression levels of EMT-related proteins (E-cadherin and vimentin). The expression level of SLC12A8 was found to be significantly higher in both NSCLC cell lines and tissues. High SLC12A8 expression was correlated with a poor prognosis in NSCLC patients. Knocking down SLC12A8 led to a significant decrease in proliferation, migration, and invasion abilities, while promoting apoptosis in NSCLC cells. Additionally, SLC12A8 knockdown resulted in decreased levels of N-cadherin and vimentin, along with increased E-cadherin expression. The results indicate that reducing SLC12A8 expression may suppress the malignant phenotype of NSCLC cells, as well as the EMT. SLC12A8 may serve as a target for the clinical management of NSCLC progression.

Key words: Cation-chloride cotransporter 9 — Cancer progression — Prognosis — Apoptosis — Targeted therapy

Abbreviations: CCC9, cation-chloride cotransporter 9; EMT, epithelial-mesenchymal transition; LUAD, lung adenocarcinoma; LUSC, lung squamous carcinoma; NSCLC, non-small cell lung cancer; STAD, stomach adenocarcinoma.

Introduction

Current data shows that non-small cell lung cancer (NSCLC) is a leading cause of cancer-related deaths worldwide due to its high prevalence rates (Li et al. 2020; Chen et al. 2021; Luo et al. 2021). NSCLC is often diagnosed in advanced stages due to its aggressive spread, frequent recurrence, and limited early detection methods. Radical resection is considered the most effective treatment for NSCLC patients, although the long-term survival rate following this procedure is notably

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low (Jie et al. 2021). The poor prognosis of NSCLC is further compounded by the lack of effective screening methods for recurrence and metastasis (Lu et al. 2020). Consequently, there is a critical need to investigate the underlying mechanisms linking NSCLC recurrence and metastasis, with the potential to revolutionize early diagnosis strategies for this disease.

The abnormal production of tumor markers by malignant cells is crucial in assessing the recurrence and metastasis of different types of cancer. For instance, LAPTM4B overexpression can enhance Nrf2-mediated stress response, leading to a poor prognosis (Maki et al. 2015). Additionally, the elevated expression level of PPM1H in cancerous tissues may serve as an independent prognostic biomarker for diagnosing NSCLC (Zhang WQ et al. 2021). In the same vein, activation of PLCγ1 was reported to facilitate the migration and invitation in NSCLC cells with EGFR mutation (Mittal et al. 2020). Furthermore, the deregulation of PVT1 has been shown to significantly influence the migration and invasion capabilities of NSCLC cells through the miR-760/IL-6 pathway (Su et al. 2020).

SLC12A8, also known as cation-chloride cotransporter 9 (CCC9), functions as a transporter of cations coupled with chloride, primarily utilizing sodium ions for the import of nicotinamide mononucleotide (NMN) into cells. Through this process, intracellular NMN forms the nicotinamide adenine dinucleotide (NAD+) coenzyme which is crucial for DNA damage repair (Grozio et al. 2019; Ito et al. 2022). Of significance, the overexpression of SLC12A8, specifically in bladder cancer, has been linked to immune infiltration (Zhang Q et al. 2021). Previous studies have suggested that SLC12A8 plays a pivotal role in inhibiting invasion and metastasis abilities of breast cancer by regulating the toll-like receptor (TLR)/nucleotide-binding domain and leucine-rich repeat containing receptor (NLR) signaling pathway (Li L et al. 2021). In addition, SLC12A8 promotes the proliferation, invasiveness, migration and epithelial-mesenchymal transition (EMT) of bladder cancer cells by activating Janus kinase/signal transducers and activators of transcription (JAK/STAT) signaling pathway (Zhang et al. 2023). Since SLC12A8 regulates cellular import of NMN, SLC12A8 could potentially regulate NAD-dependent deacetylase sirtuin-1 (Hong et al. 2020), AMP-activated protein kinase (AMPK) signaling (Han et al. 2016), and mitogen-activated protein kinase (MAPK) pathway (Assiri et al. 2019).

Although the potential of SLC12A8 as a biomarker in certain malignancies have been suggested, its implication in NSCLC progression remains unclear. Here, we explored the expression pattern of SLC12A8 in NSCLC and investigated its impact on the proliferation, migration, invasion, and EMT of NSCLC in the context of abnormal expression. The association between SLC12A8 expression level and patient prognosis was also assessed, which provides insights for potential clinical interventions in NSCLC.

Materials and Methods

Reagents

Cell Counting Kit (CCK)-8 and Bicinchoninic acid (BCA) Quantification Kit were obtained from Shanghai Biyuntian Biotechnology Co., Ltd (Shanghai, China). Fetal bovine serum (FBS), Dulbecco's Modified Eagle Medium (DMEM), pancreatin ethylenediaminetetraacetic acid (EDTA), streptomycin/penicillin, dimethyl sulfoxide (DMSO), phosphate-buffered saline (PBS), paraformaldehyde, crystal violet dye solution, and Trizol reagent were acquired from Beijing Solarbio Technology Co., Ltd (Beijing, China). Transwell chambers were procured from Shanghai Meiji Biotechnology Co., Ltd (Shanghai, China). Primescript cDNA synthesis kit and SYBR Green quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) kit were purchased from Shanghai B&C biological Co., Ltd (Shanghai, China). E-cadherin (1:1500, ab233611), N-cadherin (1:1000, ab254512), vimentin (1:1500, ab92547), and GAPDH (1:500, ab8245) antibodies were purchased from Abcam (Cambridge, UK). Lentiviral particles carrying control sh-RNA (sh-NC) or sh-SLC12A8 was purchased from Santa Cruz Biotechnology (Dallas, USA). Lipofectamine^{TM} 2000 was purchased from Shanghai Genechem co. Ltd. (Shanghai, China).

Clinical sample collection

A total of 120 NSCLC patients diagnosed at the First Affiliated Hospital of Harbin Medical University were included in this study. All the enrolled patients were diagnosed with primary NSCLC, without any prior treatment or historical records of malignancy. The tumor tissues and para-cancerous tissues were collected by surgery and examined by two independent histologists. The tissues were stored at −80°C until further analysis. The usage of clinical tissues were approved by the medical research committee of First Affiliated Hospital of Harbin Medical University, Harbin, China. All enrolled subjects provided signed written form of informed consent.

Cell culture and stable knockdown

Human normal bronchial epithelial cell line (HEB1 cells) and NSCLC cell lines (Calu-3, Calu-6, A549, and H1299) were obtained from Shanghai cell bank, Chinese Academy of Sciences (Shanghai, China). Both NSCLC and normal cells were cultured in DMEM with 10% FBS and 1% streptomycin/penicillin at 37°C with 5% CO_2 (Jinan Bo Xin Biotech-

nology Co., Ltd., Jinan, China). Lentiviral transduction was carried out when cells reached 80% confluence at an MOI of 5 in the presence of 8 μg/ml polybrene. The cells were then incubated at 37°C with 5% CO_2 for an additional 48 h and then selected with 1 μg/ml puromycin for two weeks to generate stable cell lines.

Prognostic analysis

The data on gene expression and patient profiles of NSCLC patients were retrieved and extracted from The Cancer Genome Atlas (TCGA) database. Subsequently, the expression patterns of SLC12A8 in various types of tumors were examined using the gene expression profiling interactive analysis (GEPIA) tool. Following this, the correlation between SLC12A8 expression levels and the overall survival metrics of NSCLC patients was evaluated through Kaplan-Meier (KM) plotting. A total of 120 NSCLC patients were included in this analysis, with varying parameters set for refining the survival curve linked to SLC12A8. Ultimately, the survival prognosis of NSCLC patients was assessed utilizing both univariate and multivariate COX regression analysis.

CCK-8 assay

To assess the growth potential of NSCLC cell lines, the CCK-8 assay was employed following the manufacturer's guidelines. In summary, A549 and H1299 cells in the exponential growth phase were counted and 100 μl of the cell suspension was plated in 96-well plates at a concentration of 1×10^5 cells/ml. Subsequently, CCK-8 solution (10 μl) was added to each well after 12, 24, 48, and 72 h of incubation and the cells were incubated for 2 h. The absorbance at 450 nm was measured using a microplate reader (BMG Labtech, Ortenberg, Germany), with the OD values directly correlating to the proliferation capacity of the cells.

Transwell assay

To analyze the invasion and migration capabilities of NSCLC cells, the transwell assay was conducted following the detailed protocol outlined below. In the migration experiment, cells were suspended in DMEM without FBS at a concentration of 1×10^5 cells/ml. A volume of 200 μl of this cell suspension was added to the top layer of a transwell chamber. In the lower chamber, DMEM containing 10% serum was placed, and the entire setup was incubated at 37°C in a 5% CO_2 incubator for a period of 24 h. Once the incubation was complete, the upper chamber was removed, and the cells were wiped away using a cotton swab. The cells were then stained with 0.1% crystal violet for 30 min and rinsed thrice with PBS. Subsequently, five random areas at 200× magnification were selected for cell counting under a light microscope (Leica Microsystems, Leica, Germany). In the invasion test, matrigel was added to the culture medium at a ratio of 1:8 to create a gel membrane in the upper chamber, with the remaining steps mirroring those of the migration analysis.

Flow cytometric detection of apoptosis

The flow cytometric analysis was used to detect the rate of apoptosis in NSCLC cells. The NSCLC cells in the control and knockdown groups were suspended at a density of 2×10^5 cells/ml in DMEM medium, then seeded in 6-well plates in 3 replicate. After 24-hour incubation period, the cells (1×10^5 cells/ml) were washed three times with PBS and centrifuged at 3000 rpm for 10 min. The supernatant was then removed, and 5 μl of Annexin V-FITC was added. Following a 15-min incubation at 4°C in the dark, 5 μl of propidium iodide (PI) dye solution was added for an additional 5 min in the dark at 4°C. The cell mixture was finally analyzed for apoptosis on the CytoFLEX flow cytometer (Beckman Coulter Inc., Brea, USA).

Table 1. The primer sequences used in this study

Gene	Primer	Sequence
SLC12A8	F	5'-CTA GCC TCT GCA ACA CGA CA-3'
	R	5'-CAG GAA CTG AGC AGG TGG AG-3'
E-cadherin	F	5'-GCT GGA CCG AGA GAG TTT CC-3'
	R	5'-CAA AAT CCA AGC CCG TGG TG-3'
N-cadherin	F	5'-TCA GGC GTC TGT AGA GGC TT-3'
	R	5'-ATG CAC ATC CTT CGA TAA GAC TG-3'
Vimentin	F	5'-AGT CCA CTG AGT ACC GGA GAC-3'
	R	5'-CAT TTC ACG CAT CTG GCG TTC-3'
GAPDH	F	5'-TCATTGACCTCAACTACATGGTTT-3'
	R	5'-GAA GAT GGT GAT GGG ATT TC-3'

F, forward; R, reverse.

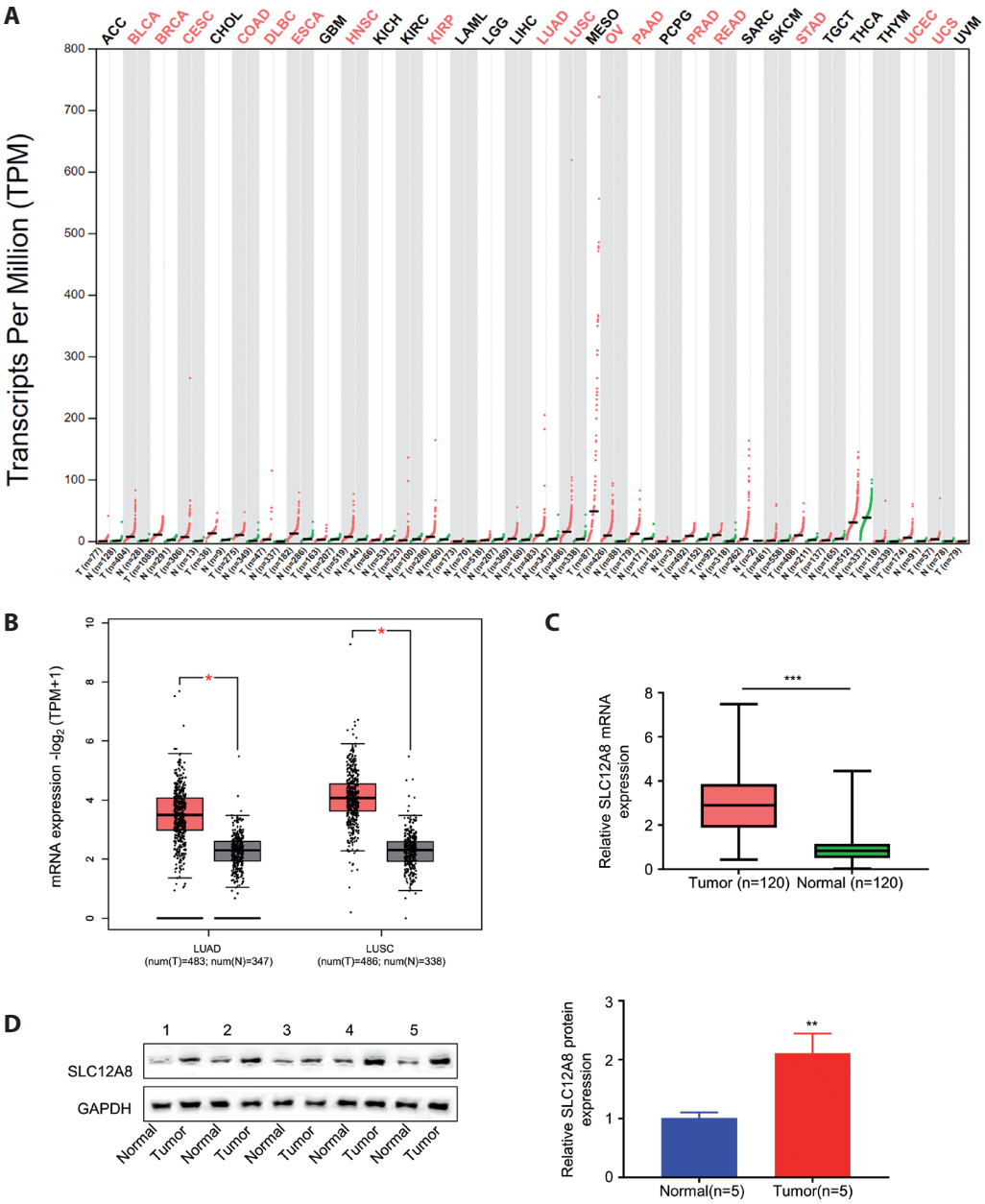


Figure 1. SLC12A8 is highly expressed in NSCLC tissues. **A.** The GEPIA analysis indicates elevated SLC12A8 expression in different tumor samples from the TCGA cohort: T: tumor, red color; N; Normal, green color. The top abbreviations stand for different cancer types, with the red font color representing the cancer type with SLC12A8 up-regulation in the tumor samples. ACC, adrenocortical carcinoma; BLCA, bladder urothelial carcinoma; BRCA, breast invasive carcinoma; CESC, cervical squamous cell carcinoma and endocervical adenocarcinoma; CHOL, cholangio carcinoma; COAD, colon adenocarcinoma; DLBC, lymphoid neoplasm diffuse large B-cell lymphoma; ESCA, esophageal carcinoma GBM, glioblastoma multiforme; HNSC, head and neck squamous cell carcinoma; KICH, kidney chromophobe; KIRC, kidney renal clear cell carcinoma; KIRP, kidney renal papillary cell carcinoma; LAML, acute myeloid leukemia; LGG, brain lower grade glioma; LIHC, liver hepatocellular carcinoma; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; MESO, mesothelioma; OV, ovarian serous cystadenocarcinoma; PAAD, pancreatic adenocarcinoma; PCPG, pheochromocytoma and paraganglioma; PRAD, prostate adenocarcinoma; READ, rectum adenocarcinoma; SARC, sarcoma; SKCM, skin cutaneous melanoma; STAD, stomach adenocarcinoma; TGCT, testicular germ cell tumors; THCA, thyroid carcinoma; THYM, thymoma; UCEC, Uterine corpus endometrial carcinoma; UCS, Uterine carcinosarcoma; UVM, Uveal melanoma. **B.** SLC12A8 expression levels in LUAD and LUSC tissues and the normal tissues from TCGA datasets: T: tumor; N; Normal. **C.** qRT-PCR analysis of the relative expression levels of SLC12A8 in 120 pairs of NSCLC tissues and adjacent non-cancerous tissues. **D.** Western blot analysis presents SLC12A8 protein levels in 5 pairs of NSCLC tumor tissues and adjacent non-cancerous tissues. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (See online version for color figure.)

RNA extraction and qRT-PCR analysis

NSCLC cells in the exponential growth phase were subjected to RNA extraction by incubating the cells in 1 ml of Trizol reagent for 30 min, followed by sequential addition of chloroform, isopropanol, and ethanol to purify and wash RNA samples. Subsequently, the quality and concentration of the extracted RNA were assessed utilizing a spectrophotometer. A total of 1 µg RNA was reverse-transcribed into cDNA with the primescript cDNA synthesis kit. The qPCR analysis was then carried out using SYBR Green qRT-PCR kit. The primer sequences utilized are in Table 1. The following cycling conditions were utilized for qPCR analysis: 93°C for 1 min; 40 cycles of 92°C for 60 s; and 70°C for 30 s. The relative mRNA expression level was analyzed using the $2^{-\Delta\Delta CT}$ method.

Western blot assay

NSCLC cells in the exponential growth phase were initially lysed utilizing the radioimmunoprecipitation assay (RIPA) buffer on ice for 15 min, and the cell lysates were centrifuged at 15000 rpm for 30 min at 4°C. Subsequently, the resulting supernatant was analyzed for protein content utilizing a BCA kit. 50 µg of denatured protein sample was subjected to a reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The blots were then transferred onto polyvinylidene fluoride (PVDF) membranes and blocked with 5% non-fat dry milk for 1 h. Subsequently, the membranes were exposed to primary antibodies (specifics of antibodies and their dilutions provided in section Reagents) overnight at 4°C. Then, the membranes were treated with horse radish peroxidase (HRP)-conjugated secondary antibody for 1 h. After the washing step with TBST buffer, signal visualization was performed using an ECL luminescent solution and the protein bands were imaged on the GelDoc imaging system (BioRad, USA). Semi-quantitative assessment of signal intensity was conducted using the ImageJ software (National Institutes of Health, USA).

Statistical analysis

The mean ± standard deviation (S.D.) were used for data expression and data analysis was conducted using Graphpad prism 5 (GraphPad Software, USA). The *t*-test and analysis of variance (ANOVA) were utilized to compare differences between two groups or among multiple groups at a significance level of $p < 0.05$. To study the correlation between SLC12A8 expression and NSCLC patient prognosis, KM curves were generated. The chi-square test was employed to analyze the correlation between SLC12A8 expression and clinicopathological data of NSCLC patients. Univariate and multivariate COX regression curves were plotted to investigate the relationship between SLC12A8 expression and various variables. Statistical significance levels were defined as follows: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

Results

SLC12A8 is highly expressed in NSCLC tissues

The study initially analyzed the expression levels of SLC12A8 in the tumor and para-cancerous normal tissues of different cancers using the GEPIA online tool, based on the datasets from the TCGA database. As shown in Figure 1A, the mRNA levels of SLC12A8 were notably elevated in the cancerous tissues of different cancer types, compared to the adjacent para-cancerous tissues. Additionally, the mRNA levels of SLC12A8 in lung adenocarcinoma (LUAD) and lung squamous carcinoma (LUSC) tissues from the TCGA database were examined. Results revealed that the mRNA levels of SLC12A8 were higher in LUAD and LUSC tissues compared to normal tissues ($p < 0.05$, Fig. 1B). Furthermore, NSCLC tumor tissues along with corresponding adjacent para-cancerous tissues were collected from 120 NSCLC patients to verify the expression pattern of SLC12A8 using qRT-PCR. The findings indicated that SLC12A8 was significantly upregulated in NSCLC tissues compared to the corresponding para-cancerous tissues ($p < 0.001$, Fig. 1C). To

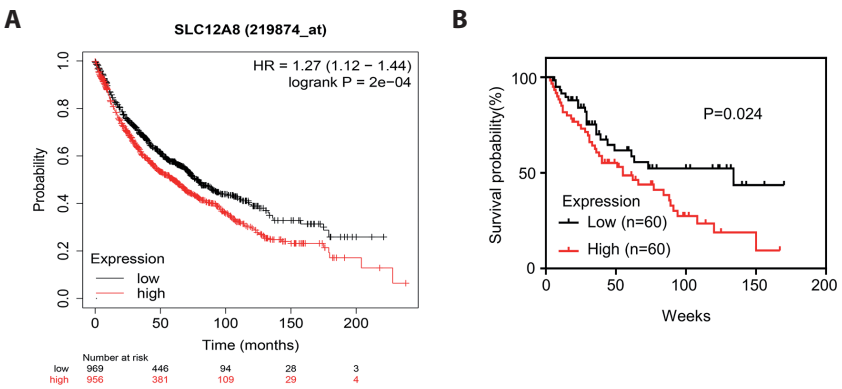
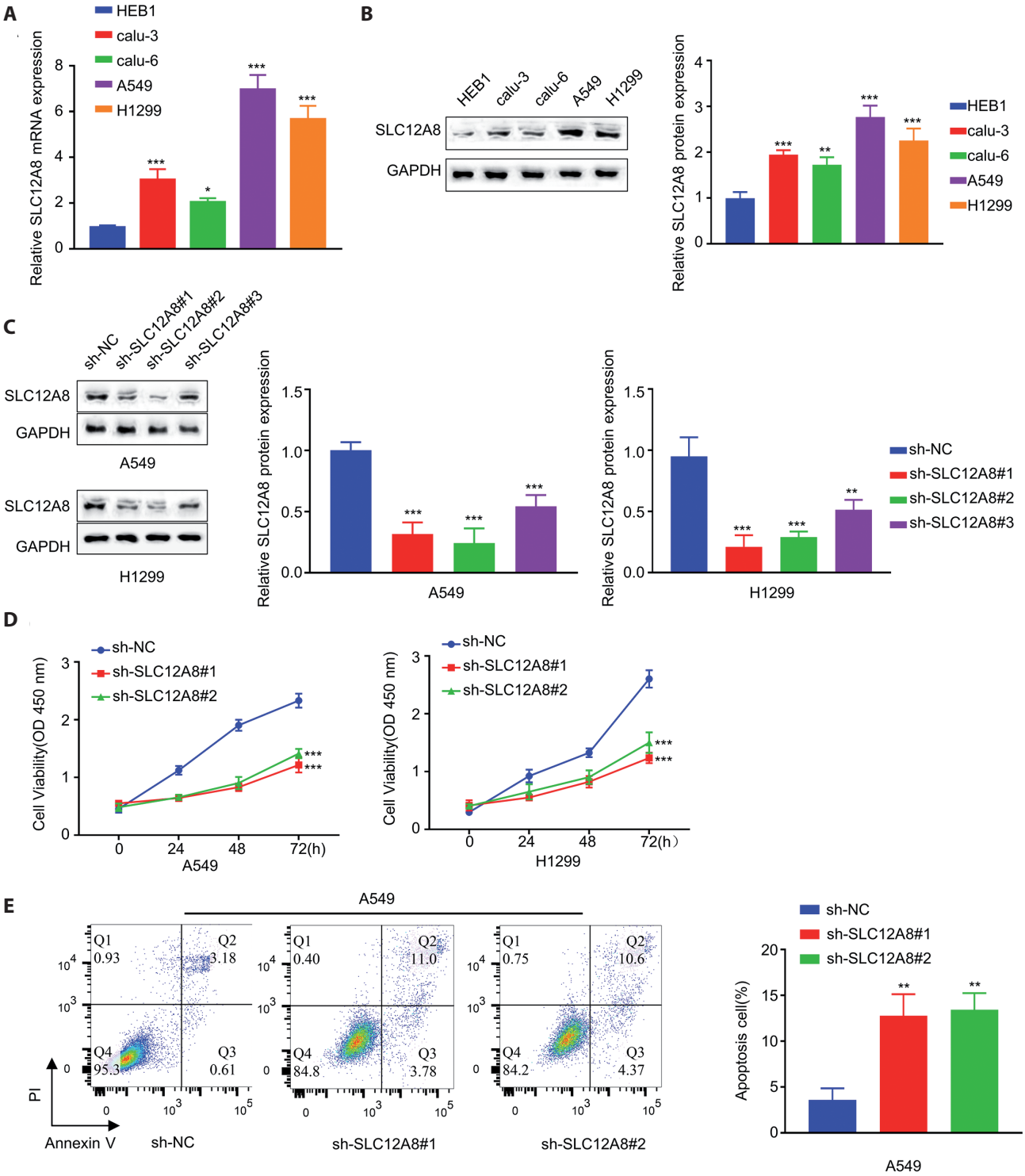


Figure 2. NSCLC patients with high expression of SLC12A8 show a poor prognosis. **A.** The KM plotter analysis shows a poor overall survival rate of cases with high expression of SLC12A8 in TCGA cohort of NSCLC patients. **B.** The KM plotter analysis indicates the shorter overall survival of NSCLC patients with high expression of SLC12A8 (recruited in this study).

demonstrate the protein expression levels of SLC12A8, Western blot analysis was performed on 5 pairs of NSCLC cancer tissues and adjacent non-cancerous tissues, which had enough tissue samples for protein extraction. There was also a higher expression of SLC12A8 protein in NSCLC tumor samples compared to matched normal tissues ($p < 0.001$, Fig. 1D).



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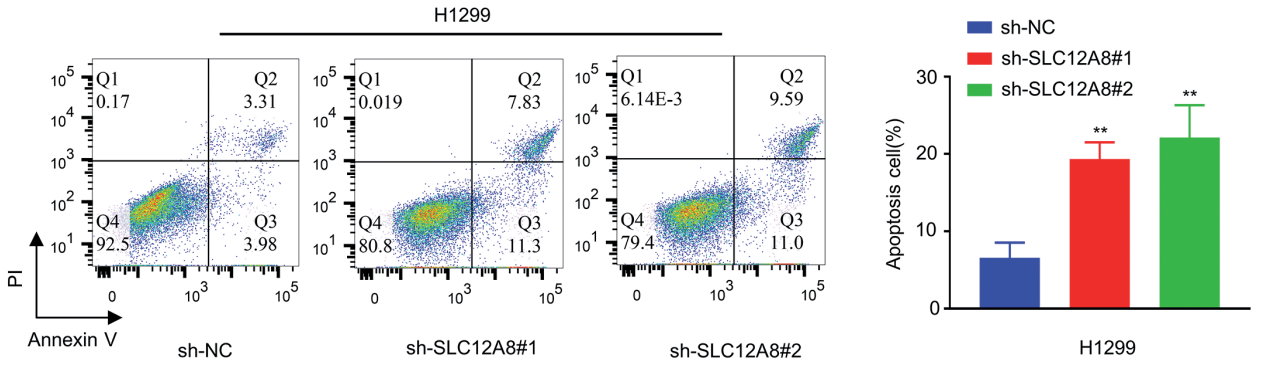

Figure 3. SLC12A8 knockdown suppresses the proliferation and induces apoptosis NSCLC cells. qRT-PCR (**A**) and Western blot (**B**) analyses measure SLC12A8 expression levels in NSCLC cell lines (Calu-3, Calu-6, A549, and H1299) and normal cells (HEB1). **C.** Western blot analysis presents SLC12A8 protein expression in NSCLC cells carrying sh-NC (negative control) or sh-SLC12A8#1, #2 and #3. **D.** The CCK-8 assay detects the absorbance values at the wavelength of 450 nm at 0 h, 24 h, 48 h, and 72 h in A549 and H1299 cells with or without SLC12A8 knockdown. **E.** The flow cytometry measures the apoptotic events in A549 and H1299 cell lines with or without SLC12A8 knockdown. $n = 3$ independent experiments. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

The high SLC12A8 expression is linked to a poor prognosis in NSCLC patients

Furthermore, we determined the relationship between SLC12A8 expression levels and the overall survival in the TCGA cohort and recruited NSCLC patients (n = 120). Notably, the individuals exhibiting high SLC12A8 expression displayed markedly inferior overall survival rates compared to their counterparts with low SLC12A8 levels (Fig. 2A,B). Moreover, we also analyzed the relationship between SLC12A8 expression and clinicopathological characteristics of NSCLC patients (Table 2). Among the attributes studied, a positive correlation was identified between SLC12A8 expression levels and tumor size, differentiation, TNM stage, and lymph node metastasis ($p < 0.001$). There was no significant correlation observed between patient age and gender. Additionally, an evaluation of potential prognostic factors for overall survival in NSCLC patients was performed through univariate and multivariate COX regression analyses. The findings from the univariate COX analysis revealed statistically significant differences in SLC12A8 expression, tumor size, differentiation, TNM stage, and lymph node metastasis ($p < 0.05$), while age and gender exhibited no significant disparities. Further, the multivariate COX regression analysis highlighted the statistical significance of SLC12A8 expression, TNM stage, and lymph node metastasis, with no significant differences observed for the other factors (Table 3, $p < 0.05$).

SLC12A8 knockdown suppresses the proliferation and promotes apoptosis in NSCLC cell lines

Prior to investigating the impact of SLC12A8 on NSCLC cell lines, the expression levels of SLC12A8 were examined in various NSCLC cell lines and normal cells. qRT-PCR and Western blot assays were used to evaluate SLC12A8 expression levels in HEB1 and four NSCLC cell lines (Calu-3, Calu-6, A549, and H1299). It was noted that the expression level of SLC12A8 was elevated in all four NSCLC cell lines compared to normal cells at both mRNA and protein levels (Fig. 3A,B, $p < 0.01$). A549 and H1299 cell lines exhibited relatively

Table 2. The summary of correlations between SLC12A8 expression levels and NSCLC clinicopathological data

Clinical characters	Total number of patients	Expression of SLC12A8		p
		low (n = 60)	high (n = 60)	
Age (years)				
≤65	53	25	28	0.581
>65	67	35	32	
Gender				
male	69	33	36	0.580
female	51	27	24	
TNM grade				
I–II	60	31	20	0.042*
III–IV	60	29	40	
Tumor size (cm)				
≤5	66	47	37	0.046*
>5	54	13	23	
Differentiation				
poor	47	12	25	
moderate	35	23	17	0.037*
high	38	25	18	
Lymph-node metastasis				
no	53	47	36	0.030*
yes	67	13	24	

* $p < 0.05$.

higher levels of SLC12A8 expression among the NSCLC cells. Consequently, these two cell lines were chosen for subsequent experiments. Three shRNAs (sh-SLC12A8#1, sh-SLC12A8#2, and sh-SLC12A8#3) were introduced into A549 and H1299 cell lines by lentiviral transfection. Western blot analysis demonstrated that, compared to the sh-NC control group, sh-SLC12A8 #1 and #2 showed efficient knockdown effect, which were selected for further functional assays (Fig. 3C, $p < 0.001$). CCK-8 assay showed that SLC12A8 knockdown led to a reduced cell proliferation in both A549 and H1299 cell lines (Fig. 3D, $p < 0.001$). Furthermore, the apoptotic events of A549 and H1299 cells in different groups were determined through flow cytometry analysis. The findings revealed that the knockdown of SLC12A8 significantly enhanced apoptosis in A549 and H1299 cells (Fig. 3E, $p < 0.01$).

SLC12A8 knockdown suppresses migration, invasion, and EMT in NSCLC cells

A transwell assay was conducted to assess the migration and invasion capabilities in A549 and H1299 cells (sh-NC and sh-SLC12A8 #1, as well as #2 groups). In comparison to the sh-NC group, the knockdown of SLC12A8 significantly suppressed the migration and invasion abilities of both A549 and H1299 cells ($p < 0.01$, depicted in Fig. 4A,B). To investigate the influence of SLC12A8 on EMT status, we detected the protein levels of various EMT markers, including the epithelial marker (E-cadherin) and the mesenchymal markers (N-cadherin and vimentin). As illustrated in Figure 4C, silencing SLC12A8 significantly reduced the expression of mesenchymal markers (N-cadherin and vimentin), while the protein level of the epithelial marker (E-cadherin) was increased ($p < 0.001$). In conclusion, these results indicate that SLC12A8 is required to support the migration, invasion, and mesennchymal status of NSCLC cells.

Discussion

According to statistical data, NSCLC cases accounts for 12.7% of all cancer cases globally. The tendency to recur and spread results in low survival rates and high mortality numbers (Jonna et al. 2019; Lu et al. 2020; Geng et al. 2021; Wang et al. 2021). Hence, it is crucial to investigate the mechanisms underlying NSCLC invasion and metastasis for the development of clinical interventions. Previous studies have reported that SLC12A8 is significantly upregulated in bladder and breast cancer, playing a significant role in promoting cancer progression (Li SL et al. 2021). In the current investigation, we found that SLC12A8 expression showed a significant increase in NSCLC tumor tissues according to the TCGA cohort data and the recruited NSCLC patients in the study. Additionally, analysis using the KM plotter revealed that NSCLC patients with elevated SLC12A8 expression had a poorer overall survival rate and survival duration. These results imply that the elevated expression of SLC12A8 may be associated with the malignant progression of NSCLC.

Furthermore, the analysis of the relationship between the expression levels of SLC12A8 and the clinicopathological data of NSCLC cases revealed a significant association with tumor size, differentiation, TNM stage, and lymph node metastasis. These findings highlight the importance of understanding the factors that either stimulate or suppress the expression of SLC12A8. Additionally, the univariate COX regression analysis demonstrated significant correlations between SLC12A8 expression levels and different clinicopathological parameters, including tumor size, differentiation, TNM stage, and lymph node metastasis. Multivariate COX regression analysis also indicated that SLC12A8 expression, TNM stage, and lymph node metastasis were independently associated with poor prognosis in NSCLC cases. Together, these findings suggest that SLC12A8 could potentially serve as a prognostic indicator for NSCLC patients, offering a potential biomarker of NSCLC prognosis.

Furthermore, Western blot analysis showed high expression of SLC12A8 protein in four NSCLC cell lines. Therefore, we hypothesized that SLC12A8 may have a crucial role in dictating the malignant features of NSCLC cells. Indeed, results from the CCK-8 assay demonstrated that reducing SLC12A8 expression could suppress the proliferation capability of both A549 and H1299 cells. Moreover, flow cytom-

Table 3. The summary of prognostic factors of the overall survival in NSCLC patients by univariate and multivariate COX regression analyses

Variables	Univariate analysis			Multivariate analysis		
	p	HR	95%Cl	*p*	HR	95%Cl
Age (≤65/>65)	0.083	1.580	0.941–2.653	0.210	2.092	1.165–3.754
Gender (male/female)	0.361	0.789	0.474–1.312	0.930	1.025	0.590–1.781
TNM grade (I–II/III–IV)	0.025*	1.828	1.079–3.099	0.033*	0.804	0.386–1.674
Tumor size (≤5/>5)	0.019*	1.986	1.183–3.333	0.165	1.692	0.805–3.557
Differentiation (poor/moderate/ high)	0.012*	1.869	1.354–2.581	0.542	1.837	1.241–2.719
Lymph-node metastasis (no/yes)	0.022*	2.620	1.436–4.781	0.021*	2.039	0.874–4.757
Expression of SLC12A8 (low/high)	0.027*	1.807	1.071–3.049	0.042*	0.650	0.289–1.465

* $p < 0.05$.

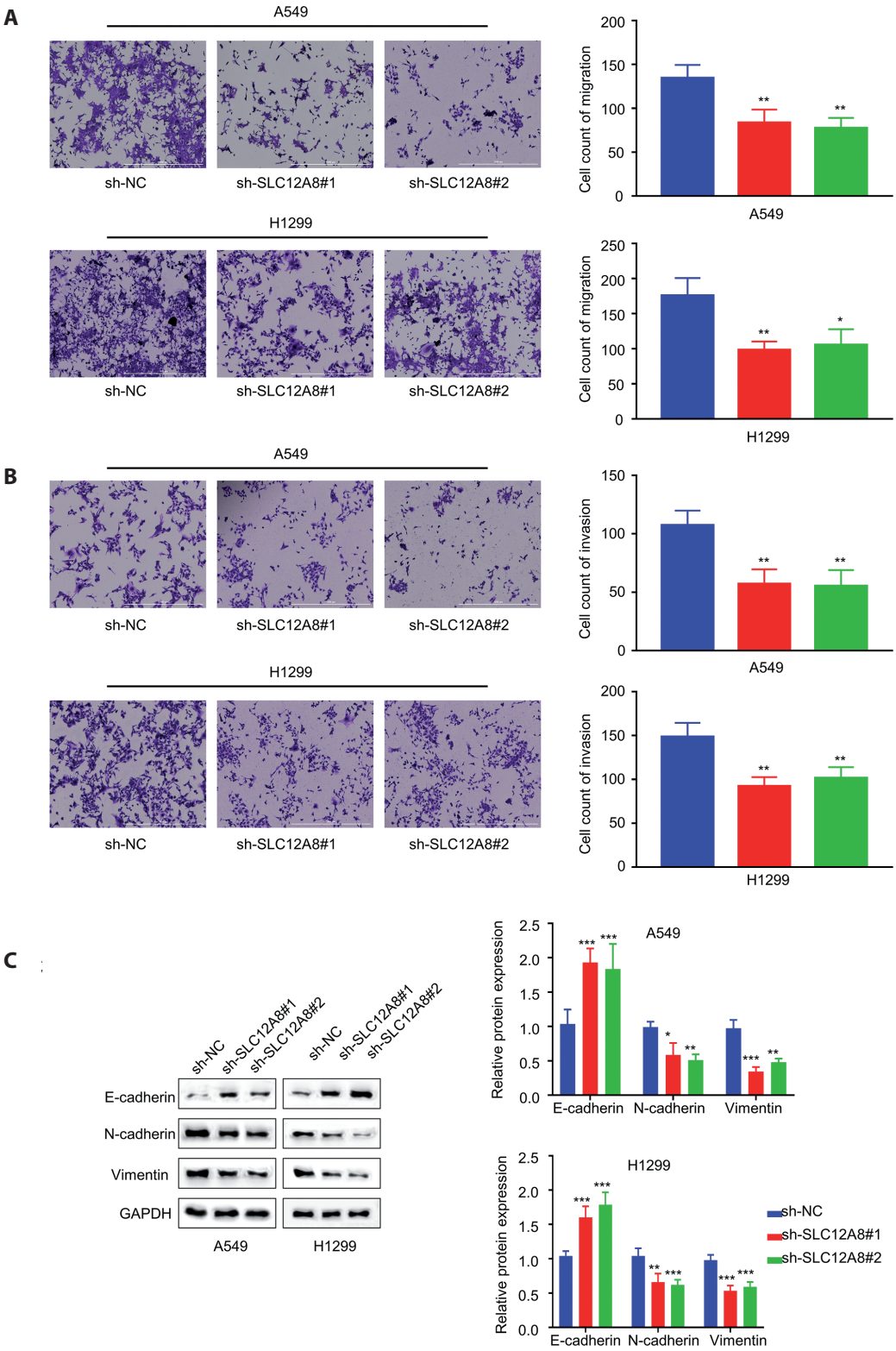

Figure 4. SLC12A8 knockdown represses the invasion, migration, and EMT in NSCLC cells. **A.** Transwell assay detects the migration ability of A549 and H1299 cells with or without SLC12A8 knockdown. **B.** Transwell assay presents the invasion abilities of A549 and H1299 cells with or without SLC12A8 knockdown. **C.** Western blot detects the protein levels of E-cadherin, N-cadherin, and vimentin in A549 and H1299 cells with or without SLC12A8 knockdown. $n = 3$ independent experiments. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

etry analysis indicated that decreasing SLC12A8 levels could enhance the rate of apoptosis in A549 and H1299 cells. These results suggest that SLC12A8 may function as an oncogene to support the survival and proliferation in NSCLC cells. Furthermore, cancer cells acquire vital abilities to invade and metastasize to enhance their survival and propagation, including under the treatment conditions (Mittal et al. 2020; Pan et al. 2021; Xu et al. 2021). These results indicate that downregulating SLC12A8 may impede the metastatic potential of NSCLC cells, which warrants future investigation in the mouse model.

EMT is a critical biological process that is intricately linked with the basic mechanisms of tumor cell invasion and migration (Chae et al. 2018). Previous research has indicated that EMT can enhance the migration and metastasis of NSCLC (Tulchinsky et al. 2019). E-cadherin, N-cadherin, and vimentin proteins, serving as key markers of EMT, are commonly found to be deregulated in tumor cells (Iderzorig et al. 2018). In one instance, an observation revealed the heightened expression of N-cadherin and vimentin, coupled with decreased expression of E-cadherin in NSCLC tumor tissues in comparison to the normal counterpart (Feng et al. 2021). Our study highlighted that the reduction in SLC12A8 expression significantly lowered the levels of the mesenchymal markers (N-cadherin and vimentin), while increasing the expression of E-cadherin in A549 and H1299 cells. This indicates a critical role of SLC12A8 in maintaining the mesenchymal state of NSCLC cells. Taken together, these findings underscore the involvement of SLC12A8 in regulating the migration, invasion, and EMT of NSCLC cells.

Despite these interesting findings, there are still some limitations in this research. A total of 120 NSCLC patients were enrolled in the study, which is a relatively small sample size. A larger and more diverse cohort would be beneficial in future study to generalize the findings across different populations. Furthermore, it is crucial to investigate whether SLC12A8 silencing could suppress tumor formation and the metastasis of NSCLC cells in the animal model. Additionally, how SLC12A8 activates EMT process to regulate the migration and invasion of NSCLC cells needs to be clarified. On top of that, there is a lack of understanding of the mechanisms governing the expression of SLC12A8. Since both genetic activation of oncogenic signaling and the alteration of epigenetic landscape account for gene deregulation in cancer (Kotsantis et al. 2016; Bradner et al. 2017; Lu et al. 2020), future work is warranted to dissect the genetic and epigenetic changes underlying SLC12A8 overexpression.

Conclusion

In conclusion, this study showed an increased expression level of SLC12A8 in NSCLC and its critical role in regulating the migratory and invasive capabilities of NSCLC cells. qRT-PCR and Western blot analyses indicated a high expression of SLC12A8 in NSCLC tissues, which is linked with unfavorable clinicopathological features in NSCLC cases. These results suggest that SLC12A8 could serve as a prognostic marker for NSCLC patients. Knocking down SLC12A8 could significantly repress cell proliferation, migration and invasion, as well as the mesenchymal state of NSLC cells. SLC12A8 may be employed as a therapeutic target to impede the malignant progression of NSCLC cells.

Ethics approval and consent to participate. The usage of clinical tissues were approved by the medical research committee of First Affiliated Hospital of Harbin Medical University, Harbin, China. All enrolled subjects provided signed written form of informed consent.

Availability of data and materials. The datasets used and/or analyzed during the current study are available from the corresponding author *via* email request.

Conlict of interests. The authors declare that they have no conflict of interest.

Authors' contributions. Jing Nie conceived and designed the experiments, Jing Nie and Xu Yang performed the experiments and wrote the paper, analyzed the data. All authors approved the final version. All authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

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