RESEARCH ARTICLE

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SLC16A13 Downregulation Contributes to Apoptosis Induction in A549 Lung Cancer Cell Line

Aysan Zeinolabedini[1], Habib Zarredar[1], Venus Zafari[2], Zahra Soleimani[1], Hamed Sabbagh-Jadid[1], Mortaza Raeisi[3]*

Abstract

Background: Lung cancer, a lethal type of malignancy in the world, has different pathological subcategories, among which NSCLC is the most common form. The complex pathogenesis of this disease has caused its treatment in advanced stages to be accompanied by many problems. Recently, the genes involved in metabolism, especially those coding for membrane transporter proteins (the solute carrier) have received attention in cancer studies. The Solute Carrier Family 16 Member 1 (SLC16A) is membrane transporters the role of which in the promotion of cancer has been revealed in recent years. This study aimed to examine the effect of *SLC16A13* low expression in A549 lung cancer cells, focusing on its role in key cellular processes such as viability, proliferation, and apoptosis. By targeting *SLC16A13*, a critical member of the solute carrier family implicated in cancer metabolism, the study search for to uncover molecular mechanisms that could inform novel therapeutic strategies for non-small cell lung cancer. **Methods:** At first, the A549 lung cancer cell line was cultured in a standard medium, and then specific synthetic *SLC16A13* sh-RNA was transfected into the A549 cell line to suppress the expression of this membrane transporter. We used MTT and flow cytometry tests to investigate the effect of reducing the expression of *SLC16A13* on the process of cell viability and apoptosis. Also, the change of gene expression was analyzed by Real-Time PCR. **Results:** In the present study, the reduction of *SLC16A13* gene expression caused an increase in the apoptosis rate and reduced cell viability in lung cancer cells. Also, *SLC16A13* suppression may induce apoptosis pathway by upregulating Bax, Caspase-3, and Caspase-9 expression while downregulation *Bcl-2* expression. Besides, it was shown that *SLC16A13* downregulation couldn't affect E-cadherin expression. **Conclusion:** *SLC16A13* may a promising target to increase cell death in lung cancer cells by inducing apoptosis pathways.

Keywords: Lung cancer- Apoptosis- *SLC16A13- Bcl-2*

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Introduction

In 2020, lung cancer has been reported to remain one of the deadliest malignancies in the world. Non-small cell lung cancer (NSCLC), comprising around 75–85% of cases, and small cell lung cancer (SCLC) are the subtypes of lung cancer [1]. In spite of the modest successes of primary detection and cure of NSCLC in the last 20 years ago, the overall mortality rate remains high (overall 5-year survival rate less than 15%), and patients have resistance to chemotherapy and radiation [2]. Therefore, it is vital to discover all the pathological and molecular aspects for designing effective treatment for lung carcinoma.

Recently, the genes involved in metabolism, especially those coding for membrane transporter proteins (the solute carrier) have been noticed in cancer. Due to their important role in cell homeostasis, they have been considered as a possible target for treatment in various malignancies, especially lung. Solute carrier (SLC) proteins, one of the largest groups of membrane proteins in humans, are present in both cell and organelle membranes [3, 4]. SLCs, which are normally involved in cargo transportation, are vital for a wide range of important physiological processes, such as drug absorption and disposition, waste removal, ion transport, and nutrient uptake [5]. Besides, it has been shown that SLCs are functionally dysregulated in some illnesses, particularly malignancies, so they can be potential targets for cancer treatment [6].

As a subfamily of solute carriers, the SLC16A family of monocarboxylate transporters (MCTs) is participated in the transmembrane transportation of monocarboxylate molecules, such as pyruvate and L-lactate. SLC16A family members also play an essential role in regulating cell metabolism reactions, such as pH homeostasis

*[1]Tuberculosis and Lung Disease Research Center, Tabriz University of Medical Sciences, Tabriz, Iran. [2]Department of basic oncology of health institute of Ege University, Izmir, Turkey. [3]Hematology and Oncology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran. *For Correspondence: raiisy@yahoo.com*

and aerobic glycolysis [7, 8]. Heretofore, 14 members have been identified for the SLC16A family, including SLC16A1 to SLC16A14. Topology studies have shown that they all have two highly conserved sequence regions located in transmembrane segment 1 (TM1) and TM5 [9]. Dysregulation of the SLC16A family member's expression, especially in *SLC16A13*, is related to the cancer progression and development. Although the precise function of different members of SLC16A family, including *SLC16A13*, through tumorigenesis is still uncertain [10]. Given the urgent essential to identify new therapeutic targets for non-small cell lung cancer (NSCLC), this research efforts on elucidating the role of *SLC16A13*, a key member of the solute carrier family related to metabolic regulation, in lung tumorigenesis. Exactly, the study examines how the low expression of *SLC16A13* impact important cellular processes, including cell viability, development, and apoptosis, by leveraging functional experiments on the A549 lung cancer cell line. This complete approach aims to reveal the molecular pathways influenced by *SLC16A13* and evaluate its potential as a promising target for NSCLC therapy.

Materials and Methods

Data validation using TCGA datasets

As the first step, we used bio-informatics analysis to determine the evaluation of *SLC16A13* in lung cancer samples. Then, using the UCSC Xena Functional Genomics Explorer, RNA-seq gene expression data for lung cancer (n = 375) and normal lung tissue samples (n = 32) was retrieved from the Cancer Genome Atlas (TCGA) database. Moreover, Kaplan–Meier survival analysis based on TCGA-LUNG and GEO datasets was carried out to investigate the correlation between *SLC16A13* expression and the overall survival rate of patients. Besides, the receiver operating characteristic (ROC) curve analysis was performed to clarify the diagnostic value of *SLC16A13* expression.

Cell culture and shRNA-mediated silencing of SLC16A13

A549 human lung adenocarcinoma cells were obtained from Pasteur Institute (Tehran, Iran). The cell culture was carried out in Roswell Park Memorial Institute (RPMI)-1640 medium, which was enriched with 10% FBS (Gibco, USA) in a cell culture incubator supplying 37°C temperature, 95% humidity, and 5% CO_2. To transfect *SLC16A13*A sh-RNA into the cells, a number of 1×10^6 A549 cells were mixed with a transfection reagent (HEPES 21 mM, NaCl 37 mM, KCl 5mM, Na2HPo4.7H2O 0.7mM, and Dextrose 6mM) and 1µg of sh-RNA in a 0.2 cuvette. Next, the mix were subjected to the Bio-rad Gene Pulser Xcell device under a transfection condition of single 140V exponential pulse with a duration of 20 ms. After transfection, the desired number of cells were mixed with 10% FBS-supplemented RPMI medium, transferred to cell culture plates and incubated for 48 h. A scrambled control sh-RNA was used as the negative control. To validate sh-RNA-mediated suppression of *SLC16A13* was measured using qRT-PCR in mRNA and protein levels, respectively. The sequence of *SLC16A13* is presented in Table 1.

MTT assay

The effect of *SLC16A13* suppression on A549 cell proliferation was investigated using the MTT colorimetric assay. After transfection, A549 cells at the density of 10×10^3 cells per well were seeded into 96-well culture plates and incubated. After incubation process for 48 h, 50µl MTT reagent (2 mg/ml; Sigma-Aldrich, USA) was added, and the plates were kept for further 4 h in the incubator. In the next step, the medium was replaced with DMSO (100 µl) to disperse the formed Formazan crystals. After keeping for 20 min at the dark place, the plates were subjected to an ELISA reader device (Biotek, ELx800, US) to read the optical density of each well based on absorbance at a of 570 nm wavelength.

Flow cytometry analysis of apoptosis

Flow cytometry analysis based on the annexin V-FITC and propidium iodide (PI) double staining was used to determine apoptosis induction upon *SLC16A13* knockdown in A549 cells. Briefly, A549 cells were seeded into 6-well culture plates at the density of 4×10^5 cells per well and after 24 h, cells transfected with *SLC16A13* sh-RNA. After incubation, the cells were harvested, washed with PBS and stained with a solution containing 200 µl binding buffer, 5 µl annexin V-FITC, and 5 µl PI(). Following the 15 min incubation at a dark place, the cells were washed, dissolved in PBS, and finally subjected to the FACSCalibur flow cytometer (). Data analysis was performed using FlowJo software version 10 (BD Biosciences, USA).

Gene expression analysis

To evaluate gene expression at mRNA levels, Real-Time PCR was employed. First, RNA extraction was done using RiboEx reagent (Gene All biotechnology, Korea). After assessment of RNA quality and quantity by Nanodrop OneC Spectrophotometer (Thermo Scientific™, USA), RNA samples were subjected to reveres transcription PCR (RT-PCR) for cDNA synthesis using 2X RT-PCR Pre-Mix (BioFACT, Korea). Next, Real-Time PCR reactions were performed using the RealQ plus Master Mix Green (Ampliqon, Denmark) in the Step OneTM qRT- PCR System (Applied Biosystem, USA). Our target genes were *SLC16A13, Bax, Bcl-2, Caspase-3, Caspase-9*, and *E-cadherin.* The *GAPDH* was used as the internal control. The primer sequences are provided in Table 2.

Table 1. The Sequence of Designed siRNA

Gene	Sequence
shRNA- *Slc16a13*	5′- GTGATTGACACAGTACGCATTCAAGAGATGCGTACTGTGTCAATCACTTTTTT-3′
	3′-CCTGGAACATTGTCTTCGTAAGTTCTCTACGAAGACAATGTTCCAGG AAAAAA-5′

Western blotting

To perform western blotting and analyze the gene expression at protein levels, the cells were washed with ice-cold PBS and then lysed using the RIPA buffer for 20 min on ice. After that, the cell lysates were pelleted by 10 min centrifuging at 12,000×g, and then the supernatants were collected. The Bio-Rad protein assay kit (Bio-Rad Laboratories) was used to determine the protein concentration for each sample, and subsequently 20 µg of each was run on 15% SDS-PAGE. After transferring to a nitrocellulose membrane, the blockage of non-specific binding was done through the incubation of membrane for a half hour at 25°C in Tris-buffered saline and 0.1% Tween-20 (TBST) with 5% skimmed milk. Next, primary antibodies, including *SLC16A13* (1:400) and *GAPDH* (1:2,000), were added to the membrane, and incubation was performed overnight at 4 °C. Then, the membrane was TBST-washed three times, and incubated with H-respective horseradish peroxide conjugated secondary antibodies for 1 h. Finally, the membrane was washed with TBST again, the bands were visualized using the Roche chemiluminescence kit (USA), and analyzed using Scion Image software (Scion Corporation, USA).

Statistical analysis

To perform statistical analyses, including T-student for two groups and One-way ANOVA for three or more groups, we used the GraphPad Prism Version 9 software (San Diego, USA). All results were expressed as mean ± standard deviation, and P values less than 0.05 were considered statistically significant.

Results

SLC16A13 was downregulated upon sh-RNA transfection in A549 cell line

To confirm *SLC16A13* suppression after transfection, Real-Time PCR was initially performed. The obtained results evidenced that *SLC16A13* mRNA expression was significantly ($P < 0.0001$) downregulated upon *SLC16A13* sh-RNA transfection in A549 cell lines. As shown in Figure 1, sh-RNA-mediated downregulation of *SLC16A13* was time-dependent, and its lowest expression was observed at 72 h time point. Besides, Western blotting assay also illustrated that *SLC16A13* sh-RNA led to the significant ($P < 0.0001$) suppression of *SLC16A13* expression at protein levels (Figure 2).

Targeting SLC16A13 inhibited A549 cell viability

We used the MTT assay to evaluate the effect of *SLC16A13* suppression on A549 cell viability. As illustrated in Figure 3, the obtained results evidenced that downregulation of *SLC16A13* led to a significant decrease in the A549 cells viability after 24 h ($P < 0.05$), 48 h ($P < 0.0001$), and 72 h ($P < 0.0001$) after transfection compared to negative control and control cells.

A549 cell apoptosis was induced upon SLC16A13 downregulation

To understanding the *SLC16A13* inhibition induce apoptosis, we done flow cytometry. The flow cytometry results demonstrated that the suppression of *SLC16A13* contributes to activating apoptosis pathways in A549 cells.

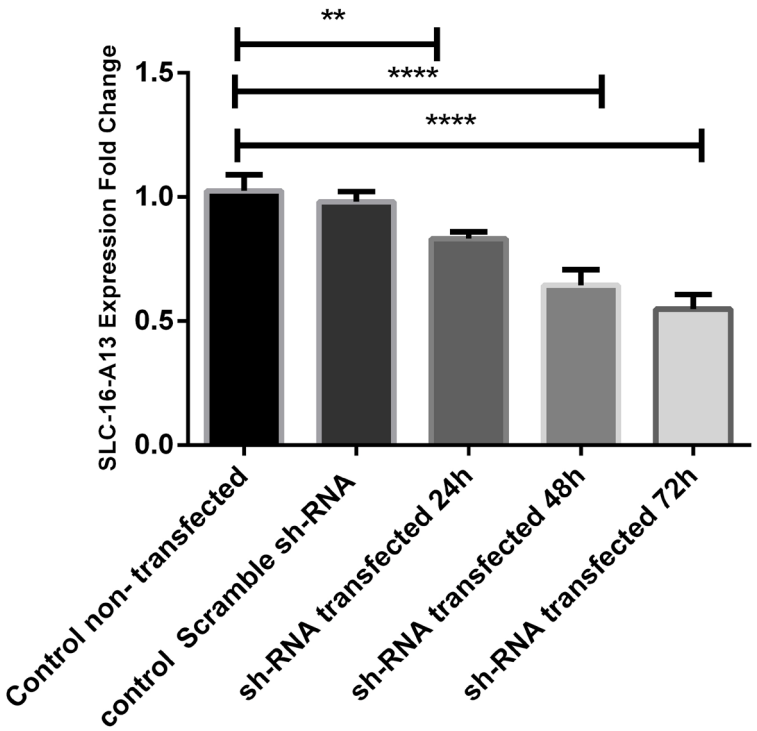

Figure 1. *SLC16A13* mRNA levels following shRNA knockdown. Quantitative PCR analyses were conducted to measure *SLC16A13* expression levels in the A549 lung cancer cell line after transfection with *SLC16A13* shRNA. The mRNA levels of *SLC16A13* were evaluated at 24, 48, and 72 hours post-transfection. The results indicate a significant, time-dependent reduction in *SLC16A13* expression in transfected cells compared to control cells treated with scrambled shRNA. Data are presented as mean ± SEM of triplicate experiments.

Table 2. Primer Sequencing

Genes	Sequences	Annealing temp (°C)
SLC16A13	Forward: 5' CCCAAACTCAGTTCCACCCTC 3'	60
	Reverse: 5' GAGCTCGGAGCTGAGCTAGT 3'	
GAPDH	Forward: 5' AAGGTGAAGGTCGGAGTCAAC 3'	60
	Reverse: 5' GGGGTCATTGATGGCAACAA 3'	
BAX	Forward: 5' GACTCCCCCCGAGAGGTCTT 3'	59
	Reverse: 5' ACAGGGCCTTGAGCACCAGTT 3'	
BCL2	Forward: 5' GAGCGTCAACCGGGAGATGTC 3'	59
	Reverse: 5' TGCCGGTTCAGGTACTCAGTC 3'	
Caspase-3	Forward: 5' ATGGTTTGAGCCTGAGCAGA 3'	59
	Reverse: 5' GGCAGCATCATCCACACATAC 3'	
Caspase-9	Forward: 5' GCAGGCTCTGGATCTCGGC 3'	59
	Reverse: 5' GCTGCTTGCCTGTTAGTTCGC 3'	
E-cadherian	Forward: 5'AAG AAG CTG GCT GAC ATG TAC GGA3'	59
	Reverse: 5'CCA CCA GCA ACG TGA TTT CTG CAT3'	

As shown in Figure 4, as time went by, the percentage of apoptotic cells significantly ($P < 0.0001$) increased.

Downregulation of SLC16A13 by siRNA transfection modulated several apoptosis-related genes

Besides, the results of Real-Time PCR also evidenced that *SLC16A13* suppression gradually upregulated the expression of apoptotic genes, including *Bax, Caspase-3,* and *Caspase-9*, whereas reduce the expression of *Bcl-2* anti-apoptotic gene (Figure 5). Also we have not detected any change in the E-Cadherin gene expression after *SLC16A13* suppression in A549 cells. Altogether, these findings implied that *SLC16A13* may contribute to lung cancer cell growth and proliferation by inhibiting apoptosis.

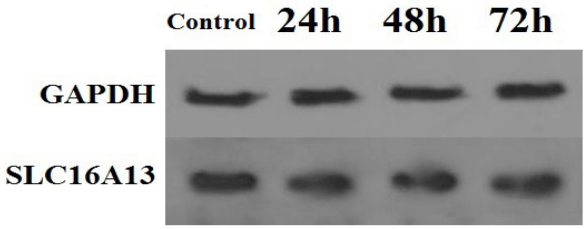

Figure 2. Decrease in *SLC16A13* Protein Levels Following shRNA Knockdown. Western blot analysis was performed on control and transfected cells at 24, 48, and 72 hours post-transfection. The results demonstrate a significant decrease in *SLC16A13* protein levels in transfected cells. The most substantial reduction was observed at 72 hours post-transfection. Anti GAPDH antibody was used as an internal control.

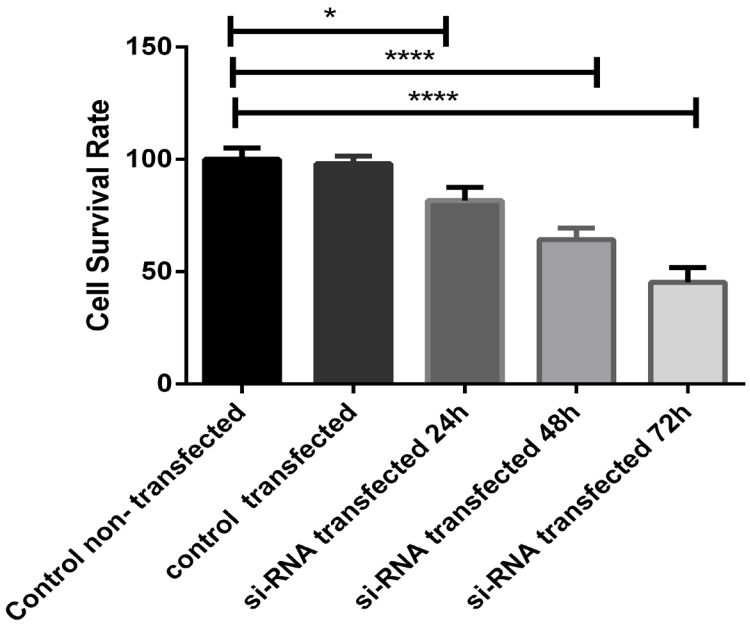

Figure 3. Knockdown of *SLC16A13* Suppresses Cell Growth in the A549 Cell Line. Cell viability was assessed using the MTT assay at 24, 48, and 72 hours post-transfection with anti-*SLC16A13* shRNA. Compared to control and blank wells, shRNA transfection significantly reduced the viability of malignant cells at all time points. Data are presented as mean ± SEM of triplicate experiments.

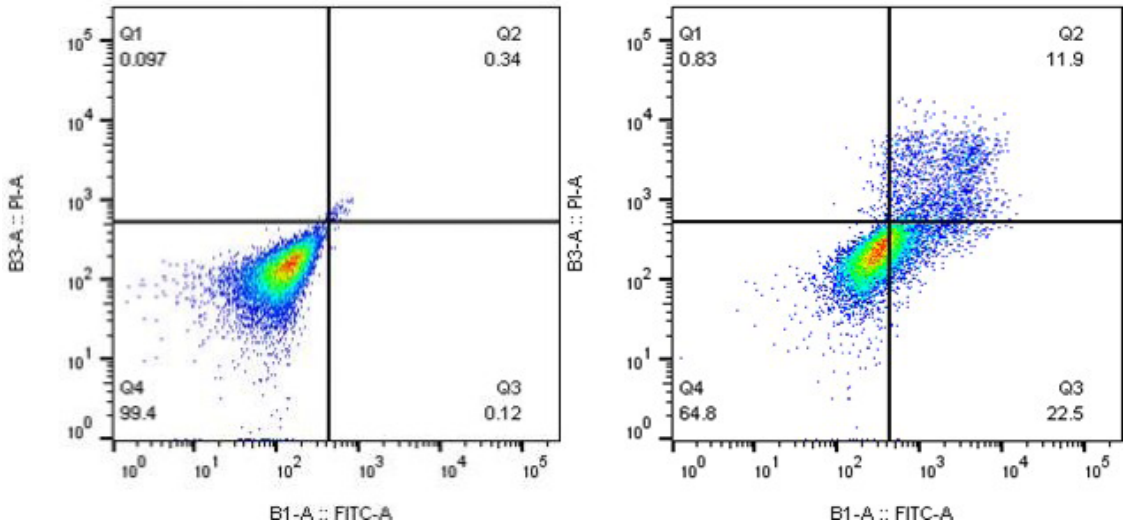

Figure 4. Effect of *SLC16A13* Knockdown on Apoptosis Levels in A549 Cells. (A) Flow cytometry analysis was used to measure apoptosis in A549 cells at 24, 48, and 72 hours post-transfection with *SLC16A13* shRNA. The results show a significant increase in apoptosis levels in transfected cells compared to controls, with the highest level of apoptosis observed at 72 hours post-transfection. Data are presented as mean ± SEM of triplicate experiments.

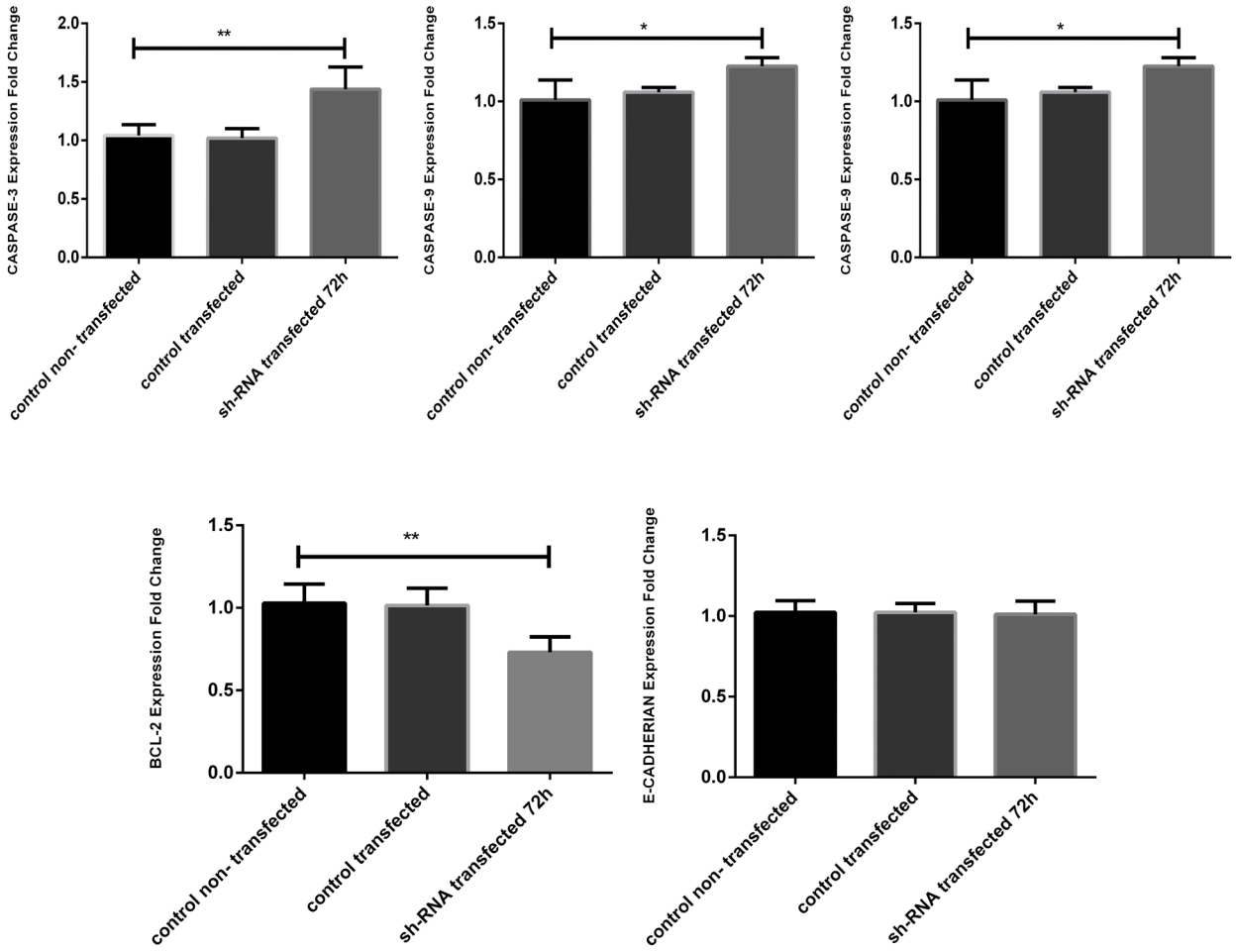

Figure 5. Suppressing effect of *SLC16A13* Gene on the Regulation of *BAX, BCL-2, Caspase-3, Caspase-9*, and *E-Cadherin* expression levels. qRT-PCR analysis was performed to measure the mRNA expression of these genes in A549 cells transfected with *SLC16A13* shRNA. Following *SLC16A13* silencing, the mRNA levels of *BAX, Caspase-3,* and *Caspase-9* significantly increased, while *BCL-2* levels significantly decreased. The reduction of *SLC16A13* had no effect on the mRNA levels of *E-Cadherin.*

Discussion

Recently some investigations confirmed that dysregulation of SLC16A family members cause a range of cancers and also can regulate cell migration, invasion, and proliferation [10]. Despite the efforts made to understand the exact function of this gene family, our understanding their roles in tumorigenesis are still unclear. The current research study was aimed to decipher the functions of one of the important members of the SLC16A family, *SLC16A13*, in lung tumorigenesis by performing a series of functional analyses.

Several studies have reported the dysregulation of the SLC family in lung adenocarcinoma and suggested that they might serve as therapeutic and prognostic targets [11, 12] because of their differently expression patterns and oncogenic roles [13].

For instance, SLC2A1 upregulation was evidenced to be correlated with lung cancer patients' poor prognosis [14]. While Leher and colleagues reported that the upregulation of another member of SLC family, SLC18A1, was correlated with high survival rates in lung cancer patients [12]. Moreover, some functional studies have shown that the dysregulation of SLC family members can be involved in the regulation of lung cancer cell invasion, proliferation, and survival [11, 14-16]. As an example, SLC38A3, an amino acid transporter, has been reported to be upregulated through metastasis of NSCLC cells, correlating with poor prognosis of the patients. SLC38A3 overexpression has been also illustrated to induce epithelial-mesenchymal transition and promote cancer cell metastasis via the regulation of transporting histidine and glutamine [17]. On the contrary, SLC25A34 and SLC46A3 have been introduced as downregulated SLC family members - the expression levels of which were correlated with lung cancer patient's prognosis, suggesting that they might function as tumor suppressor genes [18]. Furthermore, exogenous overexpression of SLC46A3 caused mitochondrial membrane potential to decrease, contributing to abnormal mitochondria morphology, and abnormal levels of copper. The suppression of cell invasion, migration, proliferation, and ameliorate sorafenib resistance was also achieved in hepatocellular carcinoma by increasing the expression of SLC46A3 [19]. More importantly, *SLC16A13* has been previously reported to be overexpressed through lung tumorigenesis, having a correlation with poor prognosis in patients. As a lactate transporter and valuable molecular target for non-alcoholic fatty liver disease and type 2 diabetes, *SLC16A13* is expressed at the plasma membrane [20]. However, its role through lung tumorigenesis needed to be further explores.

Through integrated bioinformatic analyses and results gained from our study on A549 lung cancer cell line, it was suggested that *SLC16A13* may also play a crucial role in lung cell growth and proliferation, indicating that this transmembrane transporter might be a novel molecular therapeutic target to be implicated in this malignancy [10]. First of all, analysis of lung cancer datasets in TCGA and GEO further confirmed that *SLC16A13* is overexpressed in lung cancer tumor tissues compared to normal samples. Also, its overexpression was correlated with poor prognosis and lower survival rate of patients. Next, we carried out functional experiments in vitro. After transfection of *SLC16A13* sh-RNA into A549 cells, we evaluated *SLC16A13* mRNA expression levels and protein levels at three different times, including 24, 48 and 72 h after transfection; *SLC16A13* expression levels were time-dependently reduced. Also, western blotting results showed that after suppression of *SLC16A13* expression in mRNA level, *SLC16A13* protein level reduced (72h). MTT assay showed that after transfection of *SLC16A13* sh-RNA to A549 cell line, cell viability reduced (72). Moreover, flow cytometry results indicate that suppression of *SLC16A13* gene via sh-RNA led to increased apoptosis rate in A549 cell lines. Besides, *SLC16A13* suppression revealed the growth-promoting role of *SLC16A13* in A549 cancer cells regrading to the decrease in cell survival and the increase in apoptosis rate. Moreover, Real-Time PCR results determined the expression profiles of apoptosis-related genes, including *Caspase-3*, *Caspase-9*, *Bcl-2*, and Bax after *SLC16-A13* suppression. The results showed that sh-RNA-mediated knockdown of *SLC16-A13* expression led to the high expression of Bax, *Caspase-3*, and *Caspase-9* and low expression of *Bcl-2* which is anti-apoptotic gene.

In addition, the other members of SLC16A family are also considered important tumor modulators. SLC16A1, which encodes a lactate transporter, is upregulated in melanoma cancer, and its high expression levels are correlated with poor prognosis in patients. Functionally, SLC16A1 induces immune cell infiltration and immune tolerance and promote metastasis in melanoma [21, 22]. Besides, hypoxia-mediated overexpression of SLC16A3 by transcriptional activity of hypoxia-inducible factor-1 (HIF-1) has been found to elevate colon cancer cell proliferation and tumor progression [23]. It has been also evidenced that SLC16A7 contributes to prostate tumorigenesis [24] and participates in regulating cancer cell stemness and renewal [25].

The therapeutic potential of targeting *SLC16A13* is promising. Given its role in promoting cell survival and its specific expression patterns in lung cancer, *SLC16A13* inhibitors could be developed to selectively induce apoptosis in cancer cells without affecting normal tissues. This targeted approach could enhance the efficacy of existing treatments and improve patient outcomes.

In summary, the results of current study implied that *SLC16A13*, as a member SLC16A gene family and an important membrane transporter involved in cancer metabolism, might be also a promising target in lung cancer therapy. Our results evidenced that *SLC16A13* suppression decreased lung cancer cell proliferation by activating apoptosis pathways. Therefore, conducting further in vitro and in vivo experiments in support of our findings may open new therapeutic window which is base the dependence of tumor cells on transporters participating in cancer metabolism.

Author Contribution Statement

Conceptualization: AZ and MR; methodology: HZ,

ZS, and VZ; validation: MMS and YA; Qualitative data collection and analysis: AZ, HSJ, and HZ; investigation: MR, HSJ, and ZS; writing—review and editing: AS, MR, and HZ. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement

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

Conflict of Interest Statement

The authors declare no potential conflict of interest.

Study Approval

This work was supported by a thesis grant (IR.TBZMED.VCR.REC.1400.006) from Tabriz University of Medical Sciences.

Ethical approval

All procedures performed in were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

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