SNHG6/mir-26a-5p/PIM1 Axis Could Regulate Tumorigenic Glioma Cells Behaviors

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Research Article

Keywords: Long non-coding RNAs, SNHG6, Glioma, Brain cancer

Posted Date: August 24th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-786648/v1>

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Abstract

Purpose Today, long non-coding RNAs (lncRNAs) are considered more than before. SNHG6 like many lncRNAs is proved to have different roles in human malignancies such as hepatocellular carcinoma, brain cancers and ... Glioma with a poor prognosis needs a faster way to prognosticate. Finding key molecular mechanisms of gliomagenesis is essential for producing effective drugs. We indicated a new possible molecular gliomagenesis pathway involving of PIM1 and mir-26a-5p.

Methods SNHG6 expression level was assessed in 29 brain tumor samples. Two specific siRNAs were designed and SNHG6 was silenced in A172 & U87-MG cells. Cell cycle progression, apoptosis, EMT, migration ability and survival of target cells were assessed following of SNHG6 knock-down.

Results Expression of SNHG6 in glioma tissues and target cell lines was satisfied. Following of using siRNAs, G1 arrest, mesenchymal characteristics reduction and much apoptosis of U87-MG tumorigenic cells was proved. Target cells demonstrated less proliferation and survival ability when SNHG6 was silenced. Following of SNHG6 knockdown, mir-26a-5p and PIM1 mRNA were fluctuated and U87-MG cells showed this phenomenon more prominent.

Conclusion In this research we indicated a new possible molecular pathway that could affect gliomagenesis. SNHG6/mir-26a-5p/PIM1 might be an unknown gliomagenesis molecular pathway for new treatments.

Introduction

Today, long non-coding RNAs are well known as important players of cell behavior regulators. As a big part of un-translated transcriptome of the cell, long non coding RNAs have more than 200 nucleotides in length. These long RNAs play different roles in gene regulation, apoptosis, cell cycle progression, cell senescence, epithelial to mesenchymal transition (EMT) and ... [1–5]. The role of lncRNA SNHG6 in cancer cells cycle progression and inhibition of apoptosis was proved in many cancers such as hepatocellular carcinoma and gastric cancer [6, 7]. Glioma with characteristics such as early metastasis, high malignancy and rapid progression needs effective drug(s) for treatment [8, 9].

PIM1 as an important proto-oncogene[10] could be involved in induction of malignancies in glial tissue [11, 12]. PIM1 protein as a regulator, phosphorylates its down-stream targets consecutively after activation[13].

PIM1 mRNA was considered as a target of miRNA 26a-5p in several bioinformatics prediction sites. Also, lncRNA SNHG6 was introduced as a molecular sponge of mir-26a-5p [14–16]. Sponging of miRNA 26a-5p could lead to increase of PIM1 mRNA. Therefore, lncRNA SNHG6 could act as a protective factor for PIM1 mRNA.

PIM1 mRNA stability increase is an important factor for production of more PIM1 protein in the cell. PIM1 causes apoptosis decrease [12], cell cycle progression [17], cell survival increase [18], epithelial to mesenchymal transition and cell migration increase[19].

In this study we assessed the expression of lncRNA SNHG6 in different brain cancer tissues. In the next step, possible connection of SNHG6 RNA, mir26a-5p and PIM1 mRNA was studied bioinformatically. Then, we assessed the possible role of SNHG6 in cell behaviors alteration via PIM1 by RNAi strategy.

Methods And Materials

Patients and samples

Tumor samples tissues were collected from 29 patients with brain malignancies from National Tumor Bank of Iran, which founded by Cancer Institute of Tehran University of Medical Sciences. The tissues were stored at -185°C until RNA extraction.

Cell lines

The glioma cell lines including, U87-MG and A172, were received from Pasteur institute of Iran. The cells were cultured in RPMI 1640 and DMEM media (Gibco, CA, USA) which contained 10% FBS (fetal bovine serum) in a humidified incubator with 5% CO₂ at 37 °C.

RNA extraction, synthesis of cDNA and qPCR performing

The total RNA was extracted from samples and cell lines by means of RNX plus solution (cinnaGen, Iran). The quality and quantity of the RNAs were estimated by sample visual observation on agarose gel and UV spectrophotometry. Treatment of extracted RNA with RNase free DNase was performed. First strand of cDNA was synthesized from 1 µg of extracted RNA based on protocol of manufacturer (Fermentas, Lithuania). QPCR was done using SYBR Premix Ex Taq™ II (Takara, Japan) on ABI Step One Plus real-time PCR system (Life Technologies, USA). Results of qPCR were normalized by the β-actin reference gene. The $2^{-\Delta\Delta C_t}$ method was applied for calculating of relative expressions.

Cell culture and siRNA transfection

Each cell line was cultured in appropriate medium. A172 cells were cultured in DMEM and U87-MG cells were cultured in RPMI 1640 media in humid memert incubator with 37°C and 5% Co₂ conditions.

Two specific SNHG6 siRNAs and a scramble siRNA were synthesized (Microsynth, Swiss). The transfection of siRNA was performed according to manufacturer's protocol as explained previously [20]. The efficiency of SNHG6 suppression was determined through gene expression analysis.

Cell cycle and apoptosis assays

Cell cycle and apoptosis test were performed as Alipoor , Keshavarz and their colleagues studies [21, 22].

Scratch wound Assay

SNHG6 suppression effect on migration ability of target cancer cells was investigated using scratch wound assay. The 50-60% confluent cells seeded on 12-well plate for siRNA transfection. We try to generate the same size wounds by a sterile yellow pipette tip on the monolayer cells and the wound healing speed was measured at 24 and 48 hours after creating the scratch by IMAGEJ software (NIH, Bethesda, MD, USA).

Colony forming cell assay

Colony forming assay was done for investigating the SNHG6 result on survival and proliferation ability of the target cells. To do this, the transfected cells were cultured for about 14 days and they were allowed to form visible colonies. After staining the colonies with 0.1% crystal violet (Sigma-Aldrich), they were counted and images were taken.

Immunofluorescence assay

The epithelial to mesenchymal transition (EMT) of cancer cells was studied in presence and absence of lncRNA SNHG6 by immuno-staining of E-Cadherin, Snail and Vimentin proteins. Mentioned EMT markers were investigated using Human EMT 3-Color Immunocytochemistry Kit (R&D Systems, USA) in agreement with the manufacturer's instructions as described previously [23]. The fluorescence intensity of the EMT markers was measured and analyzed by Matlab software (The MathWorks, Natick, MA, USA).

MiRNA expression assessment

Mir-26a-5p expression was quantified after SNHG6 silencing. To do this, total RNA was extracted and cDNA synthesized by the polyadenylation reaction. Then, RT- qPCR was performed using the BonYakhte kit (BON209002, Tehran, Iran) on a Rotor-Gene corbet 6000 instrument according to the previous protocol [20]. Target miRNA expression level was normalized against Snord48 as the control.

Statistical analyses

All investigations were performed three times. The relative genes expression level was calculated using the $2^{-\Delta\Delta Ct}$ method. The statistical difference between groups was calculated through the independent t-test by Graphpad prism6 software. Statistical significance level was considered at $P < 0.05$.

Results

LncRNA SNHG6 is expressed in different kinds of brain cancers.

Expression assessment in different brain cancer tissues demonstrated that SNHG6 RNA is expressed widespread among brain cancer types such as glioma (Fig. 1A).

Mir-26a-5p measurement in knock-down cells exposed that connection of lncRNA SNHG6, mir-26a-5p and PIM1 mRNA could be possible.

Knock down of SNHG6 was done in target cell lines (Fig. 1B). Measurement of mir-26a-5p level was performed by qPCR after SNHG6 knockdown. This assessment demonstrated that reduction of SNHG6 expression level and mir-26a-5p increase were arisen concurrently in transcriptome of target cells. In U87-MG cells, the level of mir-26a-5p increased more than A172 cells following of SNHG6 knockdown. Reversely, PIM1 mRNA in U87-MG cells was reduced more than A172 cells when SNHG6 had been silenced (Fig. 1C).

Knock-down U87 MG cells demonstrated much apoptosis.

Following of SNHG6 silencing, more apoptotic U87-MG cells were being seen and the difference was significant. A172 cells did not significant apoptosis increase after lncRNA SNHG6 knockdown (Fig. 2).

SNHG6 knockdown and G1 arrest of U87 MG cells could be related.

To evaluate SNHG6 effect on cell cycle progression of target cells, the cells were transfected with SNHG6 siRNAs. Following of SNHG6 knockdown, U87-MG cells showed a significant G1 arrest (Fig. 3D-F). A172 cells demonstrated non-significant G1 arrest after SNHG6 knock down (Fig. 3A-C). After SNHG6 knock down, Cyclin D1 mRNA measurement demonstrated this mRNA was reduced in U87 MG cells (Fig. 3G) more than A172 cells (Fig. 3H).

Wound healing assay demonstrated that SNHG6 could affect the migration ability of non-tumorigenic glioma cells significantly.

Migratory ability of studied glioma cells was measured following of SNHG6 knockdown. Wound healing assay demonstrated SNHG6 knockdown affected migration ability of both U87-MG and A172 cells. After SNHG6 knockdown, U87-MG and A172 cells demonstrated less migratory ability than their respective controls and this difference was significant for A172 cells (Fig. 4A).

After SNHG6 RNA knockdown, reduced survival of target glioma cells was observed.

Following of SNHG6 knockdown, the survival of transfected cells was evaluated by colony formation method. A few number of transfected cells were cultured and allowed to form colonies for 2 weeks. After staining, analyses with image.J declared SNHG6 knock-down cells had fewer colonies than their controls and the difference was more prominent for U87 MG cells (Fig. 4B).

Epithelial to mesenchymal transition (EMT) of tumorigenic glial cells could be affected by lncRNA SNHG6.

To determine the effect of SNHG6 knockdown on EMT of target cells, we measured three important proteins which are involved in EMT. E-Cadherin, Snail and vimentin proteins were assessed as known EMT markers. After siRNAs (SNHG6 and scramble) transfection, the fluorescence intensity of the immuno-stained cells was measured. Snail protein, as a known marker of mesenchymal state progression, was decreased significantly in SNHG6 silenced U87-MG cells. Subsequently, E-cadherin protein demonstrated an increased level following of SNHG6 silencing in U87-MG cells (Fig. 5A&C). Two above proteins exhibited non- significant alteration in A172 cells after SNHG6 siRNA transfection (Fig. 5B&D).

Discussion

At the present time, long non-coding RNAs, as factors that are able to alter many functions of the cells, are studding more than past. Nowadays, apoptosis, cell cycle, cell senescence, epithelial to mesenchymal transition (EMT) and ... could be altered by long non-coding RNAs [22]. The role of SNHG6 RNA in progression of hepatocellular carcinoma [6] breast [23] and gastric cancer [7] was proved. Also, role of SNHG6 RNA in gliomagenesis was investigated [24].

MiRDB, Target Scan Human and starbase v2 database predict PIM1 mRNA as a target of mir-26a-5p and sponging of this micro-RNA by SNHG6 was demonstrated in several researches [6, 14, 15]. PIM1 protein was demonstrated as an important gliomagenesis factor [11, 12]. It has been reported that PIM1 protein is up-regulated in glioma tissues. In addition, normal neuronal tissues have low to medium PIM1 protein level [12]. Therefore, determination of SNHG6 role in gliomagenesis via mir-26a-5p/PIM1 mRNA axis as a new probable carcinogenesis molecular pathway of glial tissues seemed interesting.

Of this purpose, following of SNHG6 RNA knockdown, PIM1 mRNA and mir-26a-5p expression levels were measured and the measurement indicated fluctuation of these RNAs. According to our hypothesis, SNHG6 RNA knockdown could release mir-26a-5p potentially [14, 16]. In U87-MG cells, elevated level of mir-26a-5p was confirmed after SNHG6 knockdown. Also, PIM1 mRNA was decreased in these U87-MG cells and these fluctuations were reasonable based on our hypothesis. Also, A172 cells exposed similar behavior but in a more moderate manner (Fig. 1C).

In the next phase of our research, we assessed SNHG6 knockdown effect on apoptosis of target cells. The apoptosis rate in U87-MG cells was different from A172 cells when SNHG6 siRNAs were used. U87-MG but not A172 cells demonstrated a significant increased apoptosis rate after SNHG6 knockdown (Fig. 2). PIM1 mRNA level was reduced significantly in U87-MG cells but not in A172 ones following of SNHG6 silencing. PIM1 as a proto-oncogene could prevent the cells from apoptosis and much apoptosis in U87-MG cells might relate to PIM1 level in these cells after SNHG6 knockdown [25].

Assessing cell cycle progression exposed that U87-MG cells tolerated an obvious and significant G1 arrest compared to their control ones following of SNHG6 knock down. In the same situation A172 cells demonstrated negligible and non-significant G1 arrest (Fig. 3A-F). The role of lncRNA SNHG6 was proved as a cell cycle progressive factor and also relation of cyclin D1 as a key regulator of cell cycle progression

and PIM1 is satisfied [23, 26]. So this observation could indicate possible interconnection of lncRNA SNHG6 and PIM1 that conduce to tumorigenic glioma cells cycle progression.

Cell migration ability, as an important ability for malignant cells, affects many aspects of cancer. SNHG6 role in a cancer cell migration could be different based on cell type but in many cancer cells, knock down of SNHG6 has resulted in decreased migration ability of target cells [27–29]. Cell migration ability of A172 cells reduced significantly after SNHG6 silencing and this reduction was not significant for U87-MG cells (Fig. 4A). This observation could be as a result of special protein(s) content of A172 and U87-MG cells [30].

To determine SNHG6 RNA effect on proliferation and survival of glioma cells, colony formation assay was carried out. Results of colony formation assay demonstrated that U87-MG cells formed fewer colonies when SNHG6 was destroyed and a similar result was seen for A172 cells (Fig. 4B). PIM1 that is involved in cell survival and proliferation [12, 31, 32], demonstrated a decreased mRNA level in SNHG6 silenced U87-MG cells and this observation could be connected with lower survival ability of knock-down cells.

Measurement of important EMT markers after SNHG6 silencing demonstrated that SNHG6 silenced U87-MG cells tolerated a significant reduction of Snail protein and E-cadherin increase. Alteration of these proteins level was happened with PIM1 mRNA reduction, simultaneously. Such a protein level alteration demonstrated the tendency reduction of U87-MG cells to mesenchymal state (Fig. 5A&C). In the same situation, A172 cells demonstrated non-significant alteration of EMT markers (Fig. 5B&D). In fact, for U87-MG cells, reduction of PIM1 mRNA and mesenchymal characteristics were arisen concurrently. It could demonstrate a connection between SNHG6, PIM1 and mesenchymal state of tumorigenic glioma cells. In previous studies, involvement of PIM1 in EMT was proved [33].

Taken together lncRNA SNHG6 could develop glioma by progression of EMT and cell cycle of tumorigenic glial cells which were prohibited from programmed cell death and also could be related with proliferation ability of tumorigenic glial cells. All of these observations could be of mir26a-5p sponging by lncRNA SNHG6 and PIM1 mRNA level increase.

Declarations

Funding

This research was supported by Iran National Science Foundation grant of number 93030186.

Conflict of interest

The authors declare have no conflict of interest about the article

Availability of data and material

The data underlying this article are available in the article.

Code availability

Not applicable

Authors' contributions

Conceptualization: Malek Hossein Asadi & Amin Jafari-Oliayi; Methodology: Amin Jafari-Oliayi; Formal analysis and investigation: Amin Jafari-Oliayi; Writing - original draft preparation: Amin Jafari-Oliayi; Writing - review and editing: Amin Jafari-Oliayi; Funding acquisition: Malek Hossein Asadi & Amin Jafari-Oliayi; Resources: Malek Hossein Asadi and Amin Jafari-Oliayi

Ethics approval

Not applicable

Consent to participate

Not applicable

Consent for publication

The authors declare their consent for publication of this research.

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Figures

Figure 1

SNHG6 RNA was expressed in many brain cancer types such as glioma 1A. SNHG6 reduction ratio in A172 and U87 cells is shown following of SNHG6 knockdown Fig1B. After knockdown of lncRNA SNHG6 in U87-MG cells, mir-26a-5p was increased and PIM1 mRNA was reduced subsequently. In A172 cells following of SNHG6 knockdown, mir-26a-5p showed an increased level but less noticeable than U87-MG

knock-down cells. PIM1 mRNA was reduced in A172 cells less than U87-MG cells following of SNHG6 silencing Fig1C.

Figure 2

Following of SNHG6 knockdown, apoptotic U87-MG cells number increased significantly and this observation was not seen for A172 cells following of SNHG6 knockdown Fig2.

Figure 3

A172 cells demonstrated not significant G1 arrest following of SNHG6 knockdown (Fig3A-C). Cyclin D1 as an important regulator of cell cycle progression was reduced significantly at mRNA level after SNHG6 silencing in A172 cells Fig3H. G1 arrest of U87-MG cells was seen significantly following of SNHG6 silencing (Fig3D-F). Cyclin D1 mRNA was reduced significantly in U87-MG after SNHG6 knockdown and this reduction was more noticeable than A172 cells in the same situation Fig3H.

Figure 4

SNHG6 silencing and significant reduction of migration ability were seen in A172 cells concurrently but this significant reduction was not observed for U87-MG cells following of SNHG6 knockdown Fig4A. U87-MG and A172 cells showed a significant reduction of proliferation and viability after SNHG6 knockdown and this reduction was more prominent for U87-MG cells than A172 cells Fig4B.

Figure 5

Following of SNHG6 knockdown, EMT markers assessment of A172 and U87-MG cells demonstrated that U87-MG cells had lower level of snail protein and higher level of E-cadherin protein Fig5A&C. A172 cells exposed not significant alteration of mentioned proteins following of SNHG6 knockdown Fig5B&D. Vimentin was not altered in both cell lines when SNHG6 had been silenced.