LncRNA MIAT as a Stemness-Associated Transcript Regulates the Proliferation, Migration and Metastasis of Glioma

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Research Article

Keywords: LncRNA MIAT, Glioma, Cancer Stem Cells, EMT

Posted Date: June 14th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-619425/v1

Abstract

Purpose: Glioma is the most prevalent category of brain tumors with cancer stem cells. Myocardial infarction-associated transcript (MIAT) is a long non-coding RNA (lncRNA), with altered expression in different diseases and cancers. The purpose of this study was to evaluate the functional role of lncRNA MIAT in glioma.

Methods: In this study, lncRNA MIAT expression was evaluated in the TCGA database in common cancers and glioma specimens. The expression of lncRNA MIAT was knocked down by the RNA interference method, and its effects on the characteristics of two glioma cancer cell lines, A172 and U-87MG, were investigated.

Results: The findings of the bioinformatics analysis showed an increase in the expression level of lncRNA MIAT in 12 common cancers. The expression of lncRNA MIAT was much greater in glioma tumor tissues compared to other ones. Knocking-down of lncRNA MIAT led to the reduction of proliferation of glioma cancer cells followed by cell cycle arrest at G1 phase. Furthermore, the inhibition of expression of lncRNA MIAT significantly induced apoptosis, senescence and autophagy, but limited the migration ability and Epithelial-Mesenchymal-Transition (EMT) of cancer cells. Moreover, knocking-down of lncRNA MIAT reduced the expression of stemness factors including Oct4, Sox2, and Nanog. This resulted in the upregulation of their downstream miRNAs (micro RNAs), let-7a-5p and miR-29b-3p.

Conclusion: Altogether, our findings showed that lncRNA MIAT could control proliferation, migration and metastasis of glioma cells by regulating the Nanog/ Sox2 / MAP1LC3B2/ let-7a-5p / miR-29b-3p axis. These observations proposed that lncRNA MIAT could be considered as a new oncogene in glioma.

Introduction

Cancer stem cells (CSCs) known as a sub-group of cancer cells that demonstrates stem cell characteristics containing the capacity of self-renewal and pluripotency (Czerwinska and Kaminska 2015; Zhou et al. 2016). They can either arise from stem cells with dysregulating of embryonic stem cell (ESC) associated transcription factors, or can originate from reprogrammed differentiated cells (Luo et al. 2013). Accumulating evidences demonstrated that dysregulation of ESC-associated transcription factors, like OCT4, SOX2, and Nanog could have critical roles in tumorigenesis, cancer cell migration, metastasis, and resistance to therapy (Sun et al. 2018; Tang et al. 2018). Recent reports established that not only stemness coding genes, but also ESC-associated long non-coding RNAs (lncRNAs) can contribute to the formation of CSCs (Alipoor et al. 2018; Czerwinska and Kaminska 2015).

LncRNAs are a subset of non-coding RNAs with more than 200 nucleotides length that have crucial roles in many cellular regulatory mechanisms, like proliferation, differentiation, apoptosis, and migration (Huarte 2015; Zhang et al. 2019). Moreover, their dysregulation is often observed in different diseases like cancer (Huarte 2015). In recent years, the role of lncRNAs in cancer biology has been

documented and new treatments were developed based on the regulation of these transcripts (Fatima et al. 2015).

Myocardial infarction associated transcript (MIAT) is an oncogenic lncRNA, which its expression has been altered in several cancers (Sun et al. 2018). LncRNA MIAT was reported to be expressed in ESCs. This molecule has a substantial role in maintaining the pluripotency state of ESCs through forming a regulatory loop with OCT4 (Mohamed et al. 2010). Recent reports demonstrated that lncRNA MIAT played a substantial function in various cellular processes, like differentiation, apoptosis, cellular senescence, and migration (Alipoor et al. 2018; Li et al. 2020; Zhou et al. 2020). To date extensive studies have reported dysregulation of lncRNA MIAT in various malignancies such as melanoma, ovarian, breast, and hepatocellular carcinoma (Alipoor et al. 2018; Li et al. 2020; Xiang et al. 2019; Yang et al. 2019; Zhou et al. 2020). However, its regulatory role in brain tumors is not well determined. In the present research, we explored the potential expression of lncRNA MIAT in different brain tumor tissues by quantitative Real-Time Polymerase Chain Reaction (qRT-PCR). Furthermore, to decipher its biological role, we knocked-down lncRNA MIAT expression within two brain cancer cell lines, U-87MG and A172, using the RNAi (RNA interference) technology.

Materials And Methods

Bioinformatics analysis

At first, the expression level of lncRNA MIAT was analyzed in common cancers as well as glioma by using the RNAseq data from TCGA (The Cancer Genome Atlas). In this regard, the data of each cancer were downloaded in RAW format (HTSeq-Counts). The cancers include BRCA (Breast invasive carcinoma), KIRC (Kidney renal clear cell carcinoma), KIRP (Kidney renal papillary cell carcinoma), KICH (Kidney Chromophobe), LIHC (Liver hepatocellular carcinoma), LUAD (Lung adenocarcinoma), LUSC (Lung squamous cell carcinoma), PRAD (Prostate adenocarcinoma), UCEC (Uterine Corpus Endometrial Carcinoma), STAD (Stomach adenocarcinoma), COAD (Colon adenocarcinoma), GBM (Glioblastoma multiform), and LGG (Brain Lower Grade Glioma). Using edgeR package (RRID:SCR_012802), gene expressions that were zero or close to zero were removed from the raw data by the counts-per-million (CPM) criterion. In the next step, the data were normalized using limma package (RRID:SCR_010943) based on a trimmed mean of M values (TMM) method, and the data were calculated in a logarithmic mode based on 2. The resulting expression matrix was applied to investigate the expression of lncRNA MIAT in each cancer as well as the correlation test to draw the co-expression network.

Patients

Twenty-seven brain tumor tissues were gathered from the Iran National Tumor Bank, (Tehran, Iran). They were kept at -180^{o}C until being applied for the extraction of total RNA. The clinicopathological features of the participants were accompanied by the samples obtained from the Tumor Bank.

Cell culture

Two human Glioma cell lines, U-87MG (RRID: CVCL_0022) and A172 (RRID: CVCL_0131), were used. The cell lines were taken from the national cell bank of Iran (Pasteur Institute, Tehran, Iran). The U-87MG and A172 cells were cultured in RPMI 1640 (Gibco, CA, USA) and high glucose DMEM (Gibco, Germany) medium, respectively. The mediums were enriched with 10 % fetal bovine serum (FBS), 10 µg/mL streptomycin, and 100 U/mL penicillin, and maintained in a humidified 5% CO2 incubator.

RNA isolation and gene expression analysis

Total RNA was extracted by Trizol (Invitrogen, USA) reagent following the recommended instructions. The isolated total RNA was converted to cDNA by MMLV reverse transcriptase enzyme (200 U/µL) (Fermentas, USA), RNase inhibitor (20 U), dNTP mix (1mM) along with the random hexamer primer after treating with RNase-free DNase (Thermo Scientific, USA) to remove the genomic DNA contamination as described (Scientific). Specific primers were designed using Gene Runner software, version 4.0 (Table S1). QRT-PCR was performed by SYBR Premix Ex TaqTM II (Takara, Japan) with a Rotor-Gene 6000 detection system (Corbett Life Science, Sydney, Australia). The qRT-PCR reaction was carried out with the following condition: initiation at 95 °C for 30 seconds, amplification for 45 cycles, denaturation at 95 °C for 5 seconds, and annealing and extension at 60 °C for 30 seconds (adjusted according to the Tm of the primers). The accuracy of the PCR amplification products was confirmed on a 2% agarose gel. β-Actin was as an internal control to normalize qRT-PCR analyses.

LncRNA MIAT knockdown

LncRNA MIAT SMARTpool siRNA and scramble siRNA(s) were purchased from Dharmacon (siRNA SMARTpool® IKKα, Colorado, USA). The SMARTpool contained four modified siRNAs that guaranteed the efficient knocking down of lncRNA MIAT without off-target. siRNAs were introduced to glioma cells along with Lipofectamine 2000 (Invitrogen, USA) based on the suggested protocol. In brief, 2×10^4 cancer cells were seeded per well on a 12-well plate in an antibiotic-free growth medium until reach 40-60% confluency. Then, the proper concentration of siRNAs and Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the recommended protocol was diluted in 250 µL Opti-MEM (Invitrogen, Carlsbad, CA, USA). The resulting compound was incubated for 15 minutes at room temperature, and then mixed with the cultured cancer cells. The transfected cells were further kept in a humidified 5% CO2 atmosphere, for 2 or 3 days. Then, they were used for different molecular and cellular assays. To verify the efficient knocking-down of lncRNA MIAT, its expression was measured 2 and 3 days after transfection.

Clonogenic assay

To assess the influence of lncRNA MIAT knocking-down on the clonogenic capacity of glioma cancer cells, colony formation test was done (Franken et al. 2006). After transfection of cancer cells with siRNAs, one hundred of U-87MG-MIAT-depleted and A172-MIAT-depleted cells were transferred to a new 6-well plate and maintained for 1–2 weeks at 37 °C with 10% FBS. After that, the cells (colonies) were fixed with 4% paraformaldehyde and 0.2 % glutaraldehyde, and after that, stained with crystal violet (0.1%) (Sigma-

Aldrich, Vienna, Austria) for 30 minutes. Finally, the colonies were counted in each well by ImageJ software (Version: 1.52v, NIH, USA, RRID: SCR_003070).

Cell cycle assay

Cell cycle assay was done by a nuclear propidium iodide (PI)-staining procedure(Krishan 1975). In brief, the transfected cells were detached, and washed with phosphate-buffered saline (PBS). The suspended cells were stained with PI staining solution (50 µg/mL propidium iodide, 0.1 % sodium citrate and 0.1 % Triton X 100). Then, the cells were kept for 30 minutes at room temperature without light(Fried et al. 1976). The cell cycle distribution was analyzed with a flow cytometer (Partec, Germany). The results were analyzed by flowjo 7.6.1 software (Ashland, OR, USA, RRID:SCR_008520).

Apoptosis assay

The occurrence of apoptosis was detected 48 hours after lncRNA MIAT siRNA and scramble siRNA transfection by staining the cells with Annexin V-FITC and PI apoptosis kit based on the suggested guidelines (Thermo Fisher Scientific, USA). After incubating the cells at room temperature for 20 minutes in dark, the apoptotic cells were detected with a flow cytometer (Partec, Germany). To verify the apoptosis percentage obtained from above staining, we used the CellEventTM Caspase-3/7 Green Flow Cytometry Assay Kit (Invitrogen, USA). The kit detected the activated Caspase-3/7 in apoptotic cells, which were analyzed by flow cytometry. Data analysis was carried out by flowjo 7.6.1 software (Ashland, OR, USA, RRID:SCR_008520).

Senescent cell detection

The activity of Lysosomal β-galactosidase, known as an indicator for detecting the senescent cells, was determined as explained previously (Keshavarz and Asadi 2019). Briefly, 10^4 cells were fixed with 4% paraformaldehyde and 0.2 % glutaraldehyde. Then, the cells were stained with 5-bromo-4-chloro-3-3indolyl β-D-galactoside (X-gal) that contained 1 mg/mL X-gal, 40 mM citric acid/sodium phosphate, 0.15 M NaCl, 2 mM MgCl2, 5 mM potassium ferrocyanide, and 5 mM potassium ferricyanide (pH 5.9–6.1). The cells were kept without light at 37 °C overnight and then they were visualized under a light microscope, and the stained cells were counted in three random areas.

In vitro scratch assay

In vitro scratch test was done to evaluate the effect of MIAT knockdown on cancer cell migration(Liang et al. 2007). In this regard, the transfected cells were seeded on 12-well plates until they reached approximately 90%–95% confluency. Then, the medium was removed and a cell-free gap was scratched to monolayer cells employing. The speed of wound closure was photographed in different time intervals (12 and 24 hours). To determine the migration percentage, the Image J software (NIH, Bethesda, MD, USA, RRID:SCR_003070) was applied to measure the gap distance.

Immunofluorescence staining

The expression level of E-Cadherin, Snail, and Vimentin was measured by Human EMT 3-Color Immunocytochemistry Kit (R&D Systems, USA) as explained before (Keshavarz and Asadi 2019). In summary, the 3×10^4 cells were seeded on 8 well cell culture chamber slides. Two days after transfection with lncRNA MIAT siRNA and scramble siRNA, the cells were fixed with 4% paraformaldehyde (PFA). Afterwards, they were kept in 0.3%Triton X-100, 10% normal donkey serum, and 1% BSA. Finally, the cells were incubated with monoclonal fluorochrome-conjugated primary antibodies (NL637-conjugated Goat Anti-Human E-Cadherin, NL557-conjugated Goat Anti-Human Snail, and NL493-conjugated Goat Anti-Human Vimentin). Images were taken under a fluorescence microscope (Zeiss, Germany), and 40x magnification. Quantification of the aforementioned proteins was done by measuring their color intensity using MATLAB software (MathWorks, Natick, MA, USA, RRID:SCR_013499).

Cell lysis and immunoblot analysis

Cell lysis and immunoblot were done as described previously (Alipoor et al. 2018). In summary, 72 hours after transfection of the cells with lncRNA MIAT siRNA and scramble siRNA, 10^6cells were homogenized in NP40 lysis buffer (Life Technologies, USA) and protease inhibitor cocktail (Sigma-Aldrich, USA). After that, 40 µg of total extracted protein was separated by running it on a 12% SDS-polyacrylamide gel and then it was transferred to a polyvinylidene fluoride membrane (PVDF; Roche). Then, blocking was done with Tris-buffered saline containing 5% nonfat dry milk and 0.5% Tween-20 for 1 hour at the room temperature. In the following step, membranes were incubated with primary antibody β-actin (Santa Cruz Biotechnology Cat# sc-47778 HRP, RRID:AB_2714189), SOX2 (Abcam, Cambridge, UK Cat# ab97959, RRID:AB_2341193), OCT4 (Abcam, Cambridge, UK Cat# ab19857, RRID:AB_445175), Nanog (Abcam, Cambridge, UK Cat# ab80892, RRID:AB_2150114), MAP1LC3 (Abcam, Cambridge, UK Cat# ab48394, RRID:AB_881433), and SQSTM1/p62 (Abcam, Cambridge, UK Cat# ab91526, RRID:AB_2050336) at 4 °C overnight. Next, the membranes probed with secondary horseradish peroxidase-conjugated secondary antibody (1: 2000 GE Healthcare Bio-Sciences, Chicago, IL, USA) at 4 °C overnight. Finally, the visualization of protein bands was done using ECL solution (ab65623, Abcam, Cambridge, UK) and displayed to Lu mi-Film chemiluminescent detection film (Roche, USA). The protein intensity was determined by Image LabTM Software (Biorad, USA). β-actin was considered as the internal control for normalizing the expression of proteins.

Construction of the competitive endogenous RNA (ceRNA) network

The ceRNA network was used to identify the pathways that lncRNA MIAT can act through miRNA sponging. Initially, all miRNA that bind to lncRNA MIAT were extracted using DIANA Tools (diana.e-ce.uth.gr/ LncBase v3.0, RRID:SCR_016510)(Paraskevopoulou et al. 2016). For this purpose, brain tissue, direct validation type, high confidence level and Homo sapiens species criteria were considered in the software, and the score ≥ 0.4 was used as the cut off value for the prediction analysis in the LncBase prediction section. Then, using miRWalk (http://mirwalk.umm.uni-heidelberg.de), the target genes (mRNAs) for the extracted miRNAs in the previous step, were identified. Eventually, the network was constructed using Cytoscape software (Seattle, USA, v3.3.0, RRID: SCR_003032).

Data analysis and statistics

TCGA data were analyzed using R programming language (V 4.5). Cytoscape and GlueGO software (Seattle, USA) were used for enrichment analysis, and drawing the co-expression network. Pearson correlation coefficient test was applied to investigate the correlation between lncRNA MIAT and other genes. All statistical analyses were done by GraphPad Prism 8 software (San Diego, CA, USA, RRID:SCR_002798). A one-way ANOVA followed by a Tukey posttest or Student's *t*-test were applied to specify the significance level in the experiments. *P*-values>0.05 were considered as no significant statistical level. Data are reported as mean ± Standard Deviation (SD) at triplicate independent repeats.

Results

MIAT is a cancer-associated lncRNA

The role of lncRNA MIAT as an oncogene has been established in several cancers (Shao et al. 2018; Wu et al. 2020; Zhang et al. 2020a; Zhou et al. 2020). However, it has not yet been accurately assessed in a number of other cancers, especially in glioma. In this regard, we investigated the expression level of lncRNA MIAT in 12 prevalent cancers based on TCGA data. The data revealed that the lncRNA MIAT expression significantly increased compared to normal samples in all common tumor samples except colorectal and stomach cancer (Fig 1, P<0.05). Interestingly, lncRNA MIAT expression in low-grade glioma (LGG) was significantly higher than high-grade glioma (Fig 1, P<0.001). Additionally, we examined the expression level of lncRNA MIAT in different types (Benign Meningioma, Glioma, Atypical Meningioma, and others) of brain tumor tissues using qRT-PCR. In line with the above results, lncRNA MIAT significantly upregulated in glioma cancer types rather than other brain tumor types (Fig 2a, P<0.001). Interestingly, its expression was higher in low-grade tumor samples in comparison to the high-grade ones (Fig 2b, P<0.001) (Table S2). These observations supported the notion that dysregulation of lncRNA MIAT may contribute to brain cancer initiation.

LncRNA MIAT suppression causes G1 arrest in glioma cells

To explore the potential function of lncRNA MIAT in proliferation and growth of glioma cells, we decreased its expression using smart pool MIAT siRNAs in two glioma cell lines, U-87MG and A172, which showed low and high expression levels of lncRNA MIAT, respectively (Fig. 2c). The inhibition of lncRNA MIAT transcript was verified using the qRT-PCR (Fig. 2d, P<0.01). As it was illustrated the expression of lncRNA MIAT was reduced after transfection of the cells with lncRNA MIAT siRNA. Furthermore, its suppression effects on cancer cell proliferation were investigated. Our findings revealed that knocking down of lncRNA MIAT not only restricted the ability of colony formation, but also caused G1 arrest of brain cancer cells (Fig. 2e, 2F). Besides, the expression level of two regulators of the cell cycle, Cyclin D1, and PCNA, was changed in response to lncRNA MIAT knockdown (Fig. S1 a,b, P<0.05). These results proposed that lncRNA MIAT transcript could play a crucial role in brain cancer progression through cell cycle regulation.

LncRNA MIAT knockdown induces apoptosis in glioma cell lines

Apoptosis is considered a pivotal element of different processes including normal cell turnover. Also, in several specific conditions like cancer, inhibition of apoptosis occurs (Elmore 2007; Fernald and Kurokawa 2013; Jan 2019). In this regard, we speculated that lncRNA MIAT may act as an antiapoptotic factor due to the up-regulation of lncRNA MIAT in glioma tumor tissues. Therefore, we assessed its inhibition effects on apoptosis occurrence. This was investigated either by staining the cells with Annexin-V or by measuring the activity of Caspase3/7 (Fig. 3A, 3B. $P<0.05$). In consistent with our speculation, the findings showed that the percentage of dead cells was significantly elevated following lncRNA MIAT suppression. In line with the above results, the expression of BAX, as a pro-apoptotic factor, and BCL2, an apoptosis suppressor, changed when lncRNA MIAT expression was knocked down. (Fig. S1c,d, $P<0.05$). Together, the results could introduce lncRNA MIAT transcript as an antiapoptotic factor that may play an oncogenic function in brain tumors.

LncRNA MIAT knockdown alters autophagy flux in glioma cell lines

Autophagy is known as a double-edge process in cancer that contributes to activation or restriction of tumor progression. Given the above results, we hypothesized that lncRNA MIAT may involve in autophagy regulation. Therefore, we determined the expression level of autophagy markers after lncRNA MIAT knockdown. Analysis of the autophagy associated proteins (MAP1LC3 and SQSTM1/p62) expression showed that knocking down of lncRNA MIAT transcript reduced the Microtubule-associated proteins 1A/1B light chain 3B (MAP1LC3B1) expression, while the expression level of MAP1LC3B2 was not statistically significant. On the other hand, lncRNA MIAT inhibition induced the expression of p62/SQSTM1 (Fig. 3c).

LncRNA MIAT knockdown increases cellular senescence in glioma cell lines

Accumulating evidences have established that cellular senescence is a barrier for cancer progression (Ohtani et al. 2009; Prieur and Peeper 2008; Sacco et al. 2021). Therefore, we encouraged to determine the occurrence of senescence after lncRNA MIAT depletion by β-X-gal staining. Our findings showed that a portion of senescent cells significantly increased following lncRNA MIAT down-regulation in brain cancer cells (Fig. 4a). In confirmation of the above findings, the expression level of CDKN2A ($p16^{INK4a}$), as an aging biomarker, was altered as expected (Fig. 4a).

LncRNA MIAT inhibition results in a reduction of migration and EMT in glioma cells

Given the up-regulation of lncRNA MIAT in glioma, we sought to investigate if it may have a regulatory role in cell migration and Epithelial-Mesenchymal-Transition (EMT) of glioma cells. In this way, wound scratch test was done to evaluate the capacity of migration of cancer cells in response to lncRNA MIAT knockdown. LncRNA MIAT silencing leads to reduction of capability of migration of cancer cells with compared to those were treated with scramble siRNA (Fig. 4b, $P<0.05$).

Furthermore, EMT induction occurs during cancer progression that leads to metastasis(Jolly et al. 2017; Vu and Datta 2017). Hence, we assessed the effects of lncRNA MIAT silencing on EMT status. As expected, the expression of E-cadherin increased while the expression level of Snail and Vimentin decreased after MIAT suppression (Fig. 5a and 5B). Overall, these observations suggested that lncRNA MIAT transcript may play a potential regulatory role in induction of EMT and consequently in metastasis of the brain cancer cells.

Knocking down of lncRNA MIAT downregulates stemness factors

In the present research, the expression level of ESC-associated transcription factors including OCT4, SOX2, and Nanog was explored when lncRNA MIAT was restricted. We found that their expression significantly decreased at protein (Fig. 6a,b,c,d) and mRNA (Fig. 6e,f) levels. This finding verified the regulatory role of lncRNA MIAT in the formation of the CSCs.

MIAT knockdown affects miRNAs expression through the ceRNA network

Since lncRNA MIAT expression was able to influence autophagy and stemness factors, the mechanism of this effect was evaluated through the ceRNA network. We found that miR-29c-3p, let-7c-5p, let-7a-5p, let-7b-5p, let-7f-59 and miR-29b-3p could be sponged by lncRNA MIAT, while the targets of these miRNAs were autophagy and stemness factors (Fig. 6g). For more detailed examination, let-7a-5p and miR-29b-3p expression that targeted Nanog, SOX2 and MAP1LC3B2, was evaluated following lncRNA MIAT knockdown. The results revealed that the expression of these miRNAs increased following depletion of lncRNA MIAT (Fig. 6h, $P<0.01$). These results suggested that lncRNA MIAT might have a role in the regulation of autophagy mechanism and the expression of stemness factors through the ceRNA network.

Discussion

Recent findings have specified lncRNAs as functional modulators of various biological mechanisms, such as growth and metastasis of human tumors (Malek et al. 2014; Schmitt and Chang 2016). However, the role and mechanism of lncRNA MIAT in brain cancer have not thoroughly been clarified. In this research, we obtained a novel evidence about the potential function of lncRNA MIAT in brain cancer progression. We observed that the expression of lncRNA MIAT was upregulated in glioma tissue samples in comparison to adjacent non-tumor samples. This data was obtained from TCGA data as well as our expression analysis on brain tumor tissues. Moreover, we found that lncRNA MIAT expression was higher in low-grade glioma tissues rather than high-grade ones. This observation suggested a protective role for lncRNA MIAT in glioma. Moreover, low expression (down-regulation) of lncRNA MIAT in high grade tumors also showed that it could be a negative regulator of factors limiting neural cell growth. Therefore, high grade tumors need its low expression to obtain oncogenic characteristics and escape the neural cell growth restrictions.

To evaluate the functional role of lncRNA MIAT in glioma progression, its expression was inhibited/silenced in two glioma cell lines, U-87MG and A172, using lncRNA MIAT siRNAs. Analysis of the

suppression effects of lncRNA MIAT on proliferation, apoptosis, autophagy, cellular senescence and migration, as well as the expression of cancer stem cells (CSCs) related genes, was assessed. The data showed that lncRNA MIAT suppression could lead to a reduction in the proliferation ability of brain cancer cells and subsequently caused cell cycle arrest at G1 phase, through Cyclin D1 and PCNA regulation (Charles 1996). Previous reports documented that lncRNA MIAT promotes cancer cell proliferation in different cancer types including breast (Alipoor et al. 2018), melanoma (Yang et al. 2019), and esophageal (Zhang et al. 2020b) cancers. In line with the previous studies, results from this study verified the vital role of lncRNA MIAT in regulation of proliferation and cell cycle progression of glioma cancer cells.

There are different defensive mechanisms such as apoptosis, autophagy, and cellular senescence when a cell faces a stress(Sui et al. 2015). These are natural barriers, restricting malignant cells from surviving and spreading(Campisi 2013). In general, autophagy causes an interference with the induction of apoptosis in cancer cells as well as it can act as a pro-senescent process in cancer cells (Fitzwalter et al. 2018). However, damaged autophagy can trigger senescence in muscle stem cells (García-Prat et al. 2016) or breast cancer cells (Vijayaraghavan et al. 2017). These observations show that the consequence of autophagy is highly associated with the type and context of the cancer cells (Long and Ryan 2012; Pawlowska et al. 2018).

Based on our results, the knockdown of lncRNA MIAT could induce apoptosis in brain cancer cells, suggesting its substantial function in tumorigenesis and tumor progression of brain cancer. Furthermore, autophagy was evaluated following lncRNA MIAT knockdown. The findings revealed that lncRNA MIAT could influence the expression of autophagy markers, such as MAP1LC3 and SQSTM1/p62, and increase malignancy through this pathway.

The microtubule-associated protein 1 light chain 3 (LC3) is the key autophagy factor regulating main stages in the autophagic system including the development of the autophagic membrane, recognition of the autophagic cargo, and integration of the autophagic lysosome (Kuma et al. 2004; Pankiv et al. 2010). Moreover, the expression of Sequestosome1 (p62/SQSTM1), which is eliminated through autophagy, is the other autophagy marker (Komatsu et al. 2012). We found that following lncRNA MIAT restriction, the LC3 (MAP1LC3B1) downregulation and the SQSTM1 upregulation was occurred leading to the acceleration of the autophagy flux formation. Our results suggested that lncRNA MIAT transcript could be involved in brain cancer progression mainly through limiting the cellular autophagy.

In our previous research, we have reported the biological function of lncRNA MIAT in regulation of cellular senescence(Alipoor et al. 2018). Therefore, we evaluated the impacts of lncRNA MIAT suppression on the senescence status of the two studied glioma cell lines, U-87MG and A172. In line with our previous report, the percentage of senescent cells was elevated following knockdown of lncRNA MIAT in glioma cell lines. Therefore, we concluded that lncRNA MIAT transcript could act as a factor that restricts cellular senescence, leading to promotion of glioma.

In the next phase of our study, we evaluated the role of lncRNA MIAT in the migration ability of cancer

cells. According to our findings, lncRNAMIAT knockdown caused a reduction in the migration ability of brain cancer cells. Analysis of the expression of EMT markers showed that lncRNA MIAT knocking down could limit EMT as well as migration ability. Together, these observations suggested that the lncRNA MIAT transcript could contribute to the EMT and migration of glioma cells.

Despite the fact that several studies have deciphered the signaling mechanisms that control the stemness and self-renewal capacity of glioma stem cells (GSCs) (Maiuri et al. 2011; Zhu et al. 2011), the function of lncRNAs in GSCs remains largely unknown. It has been reported that lncRNA MIAT suppression facilitated osteogenic differentiation of human adipose-derived stem cells (hASCs), implying its function as an endogenous suppressor of stem cell osteogenic differentiation (Jin et al. 2017). In this regard, we examined the expression of the stemness associated factors (OCT4, SOX2 and Nanog) following lncRNA MIAT knockdown. The results revealed that the expression of OCT4, SOX2 and Nanog was decreased at both mRNA and protein levels. Previous reports demonstrated that lncRNA MIAT was expressed in embryonic stem cells, and it is mediated by either ES-associated transcription factors, OCT4 and Nanog, or self-regulation of pluripotency, and developmental properties(Mohamed et al. 2010). Furthermore, constitution of a regulatory loop between lncRNA MIAT and OCT4 in embryonic stem cells was reported to regulate the pluripotency status of the cells (Mohamed et al. 2010). Altogether, these data could confirm the role of lncRNA MIAT in pluripotency establishment and maintenance throughout reprogramming in pluripotent stem cells.

Moreover, the ceRNA network showed that changes in the amount of these factors can occur through sponging of miRNAs by lncRNA MIAT. According to our previous studies, stemness-related transcription factors and miRNAs have differential expression profiles in multiple human malignancies and play critical functions in tumorigenesis and metastasis (Anastasiadou et al. 2018; Anokye-Danso et al. 2012). For example, SOX2 regulates OCT4, miR-302, and let-7 expression, which have vital roles in self-renewal promotion and dedifferentiation of embryonic stem cells (ESCs) (Peng et al. 2018). Our results showed that lncRNA MIAT can sponge miRNAs including let-7a-5p and miR-29b-3p bioinformatically, and it can affect their expression. Let-7 is considered as a tumor suppressor and it has anti-proliferative activities by suppressing several oncogenes and modulating key regulators of the cell cycle, cell differentiation, and apoptotic pathways (Wang et al. 2012). Therefore, these regulatory roles support its down-regulation following lncRNA MIAT suppression. It has been reported that let-7 depletion contributes to carcinogenesis by increasing stemness factors (Chirshev et al. 2019). On the other hand, the role of miR-29-3p in stemness inhibition was reported in human glioblastoma multiform (Chung et al. 2015). Moreover, the results of our previous study revealed the existence of miR-29a, miR-29c and miR-29b target sequence on lncRNA MIAT, which suggested that it could modulate their expression (Alipoor et al. 2018). Based on these data, miR-29b-3p expression level was altered significantly following lncRNA MIAT down regulation. A study showed that in giloblastoma multiform-initiating cells, miR-29a and miR-29b, by targeting Mcl-1 (Myeloid cell leukemia-1), could increase apoptosis (Yan et al. 2015). Therefore, we speculated that the MIAT/ Nanog/ SOX2 / MAP1LC3B2 complex might regulate the glioma cancer cells proliferation by mediating the expression of let-7a-5p and miR-29b-3p.

In summary, since the expression of lncRNA MIAT altered in different prevalent cancers, especially glioma, it could be introduced as an oncogenic lncRNA in glioma by regulating stemness associated factors. Furthermore, it could be considered as a potential biomarker and therapeutic target in diagnosis and treatment of glioma. Therefore, it is suggested to clinically further evaluate the lncRNA MIAT application as a biomarker in large-sample studies.

Declarations

‘Consent to participate’

Funding

None

Conflicts of interest/Competing interests

The authors declare that they have no conflict of interest.

Ethics approval

This study was approved by the Ethics Committee of Graduate University of Advanced Technology, Kerman, Iran. All procedures performed in studies involving human participants involving human participants 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.

Consent to participate

A written informed consent was obtained from all individual participants included in the study.

Consent to publish

Not applicable

Data availability statement

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

Code availability

Not applicable

Authors' contributions

All authors contributed to the study conception and design. Material preparation and data collection were performed by Farzane Amirmahani, Malek Hossein Asadi and Sadeq Vallian. Data analysis was performed by Farzane Amirmahani. The first draft of the manuscript was written by Farzane Amirmahani. Malek Hossein Asadi and Sadeq Vallian commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Figures

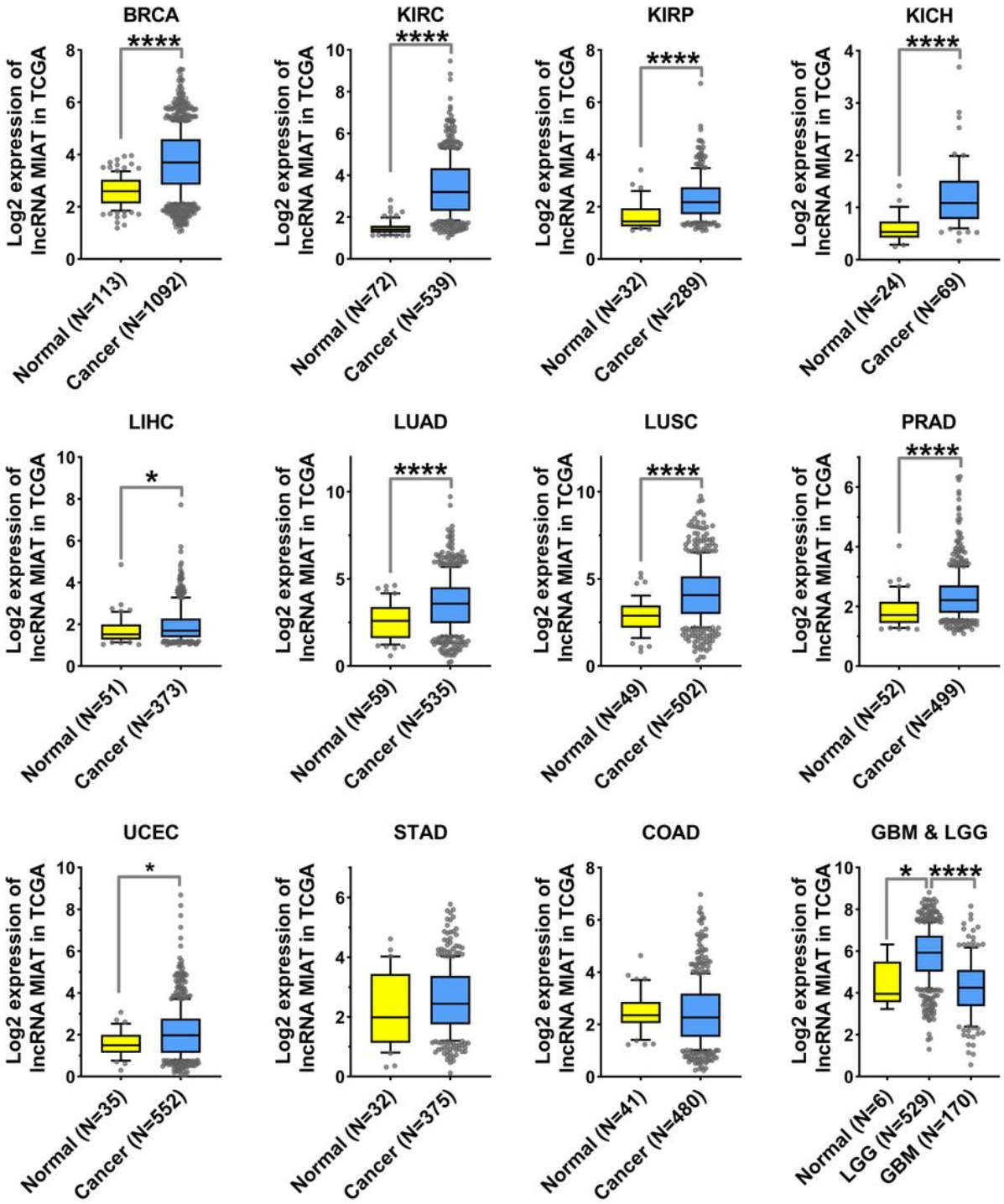

Figure 1

LncRNA MIAT was up-regulated in prevalent cancers. TCGA database was used to pan-cancer analysis, and the expression of lncRNA MIAT in different cancers was evaluated. Normalized and logarithmic data based on 2 for each cancer were used to plot the graphs (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001)

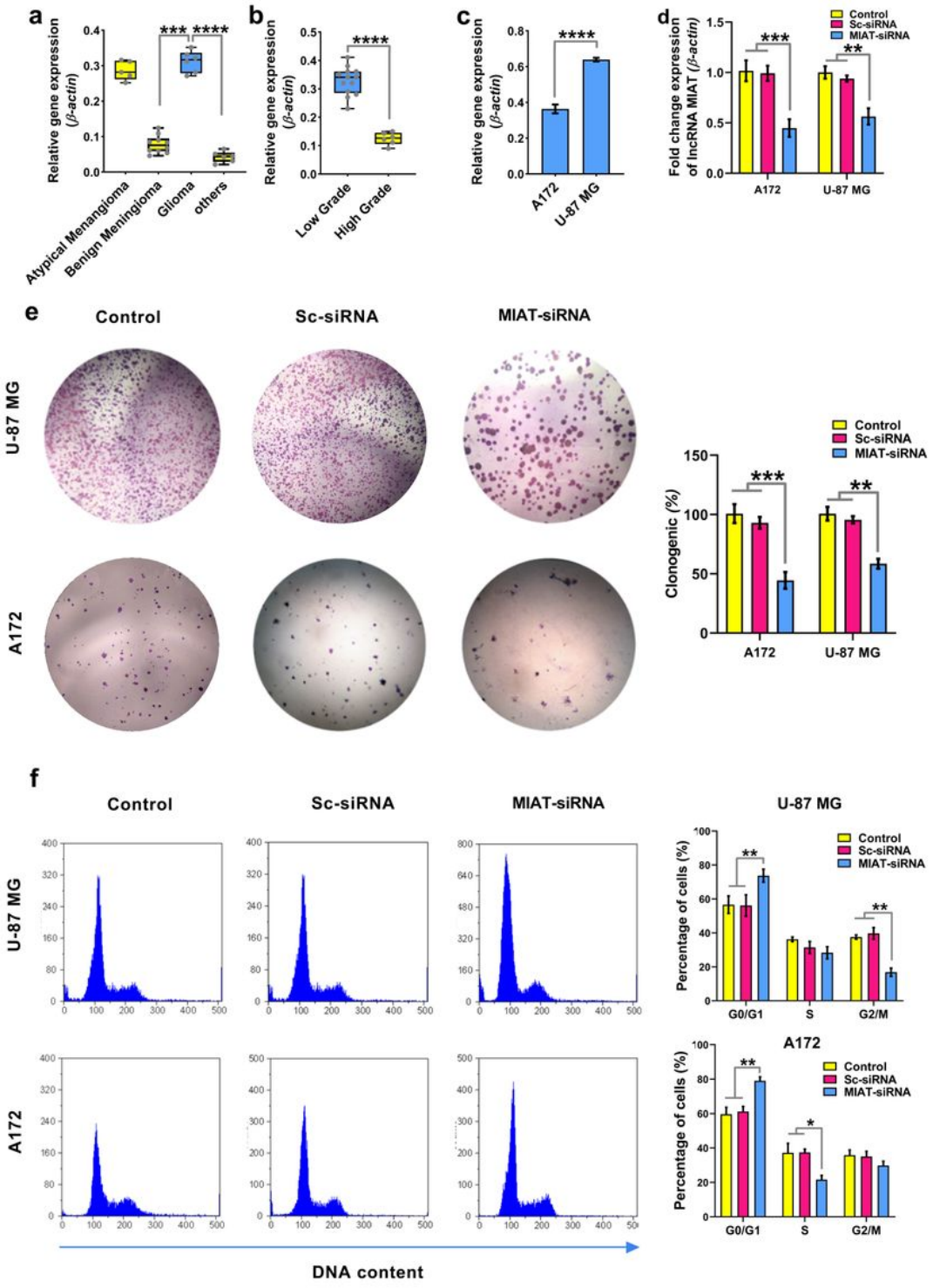

Figure 2

LncRNA MIAT knockdown restricted the clonogenic capacity and cell cycle progression of glioma cancer cells. (a) LncRNA MIAT was up-regulated significantly in human glioma in comparison to the other brain cancer tissue types. The relative lncRNA MIAT expression was normalized against β-Actin. Statistical analysis was done using paired t-test. (b) LncRNA MIAT expression was higher in low grade tumor samples rather than high-grade ones. (c) LncRNA MIAT expression was detected in two glioma cell lines,

U-87MG and A172. (d) Knockdown of lncRNA MIAT in U-87MG and A172 cells by si-MIAT was confirmed using qRT-PCR. Statistical analysis was done by one-way ANOVA with Tukey's post hoc test. (e) The clonogenic ability of glioma cancer cells with lncRNA MIAT suppression restricted in comparison to the cells transfected with scramble siRNA. (f) LncRNA MIAT transcript suppression led to G1/S arrest in U-87MG and A172 cells. Data are demonstrated as mean ± SD at triplicate independent repeats (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001)

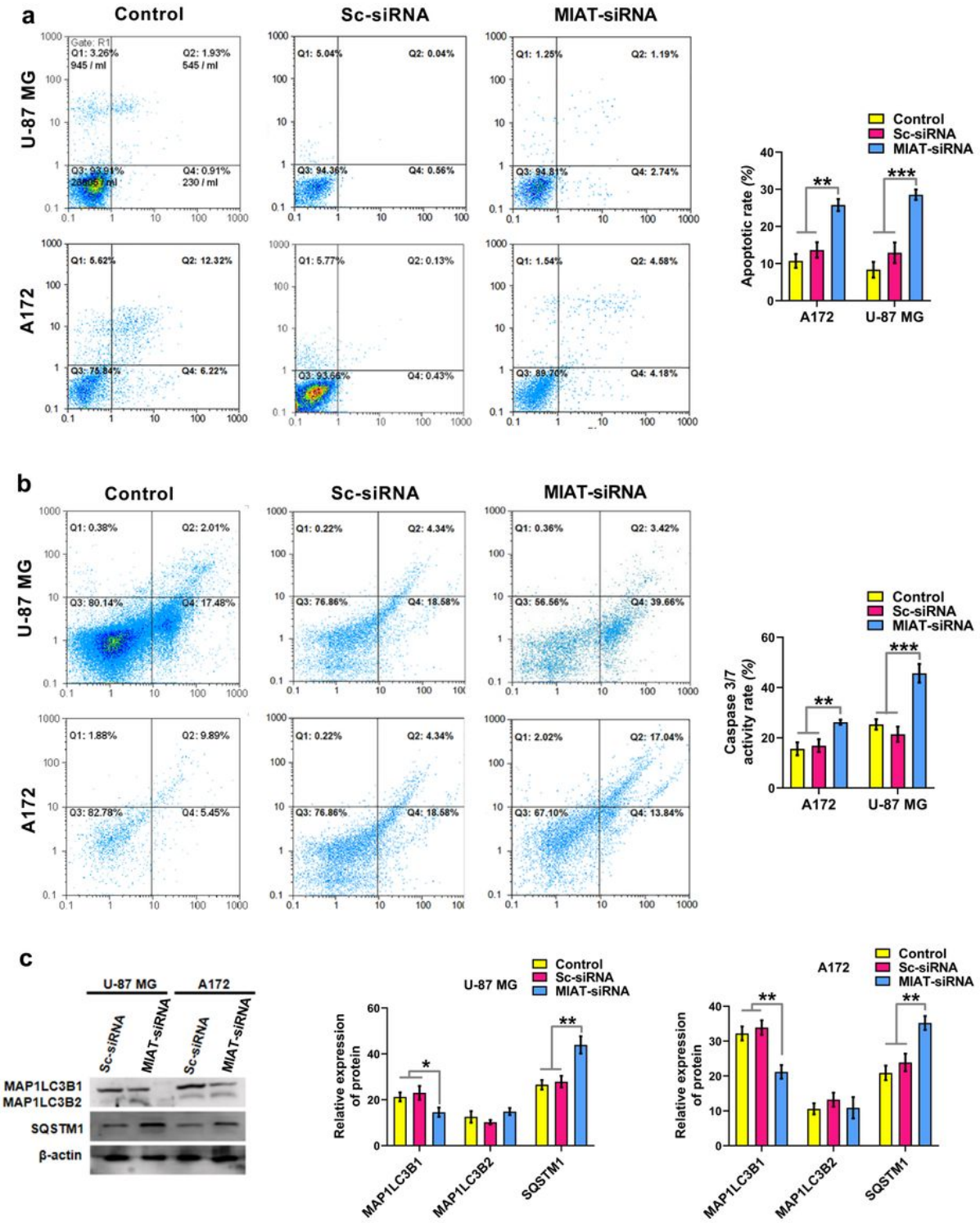

Figure 3

LncRNA MIAT knockdown changed apoptosis and autophagy flux in glioma cancer cells. (a) LncRNA MIAT knockdown dramatically caused apoptosis in glioma cancer cells, especially in A172 cell line. This was determined not only by using Annexin V/PI (propidium iodide) staining but also by (b) measuring the activity of Caspase-3/7 following lncRNA MIAT inhibition. (c) The expression of MAP1LC3-1/2 and SQSTM1/p62 altered after lncRNA MIAT down-regulation at the protein level. Data are represented as mean ±SD based on at triplicate independent replicates

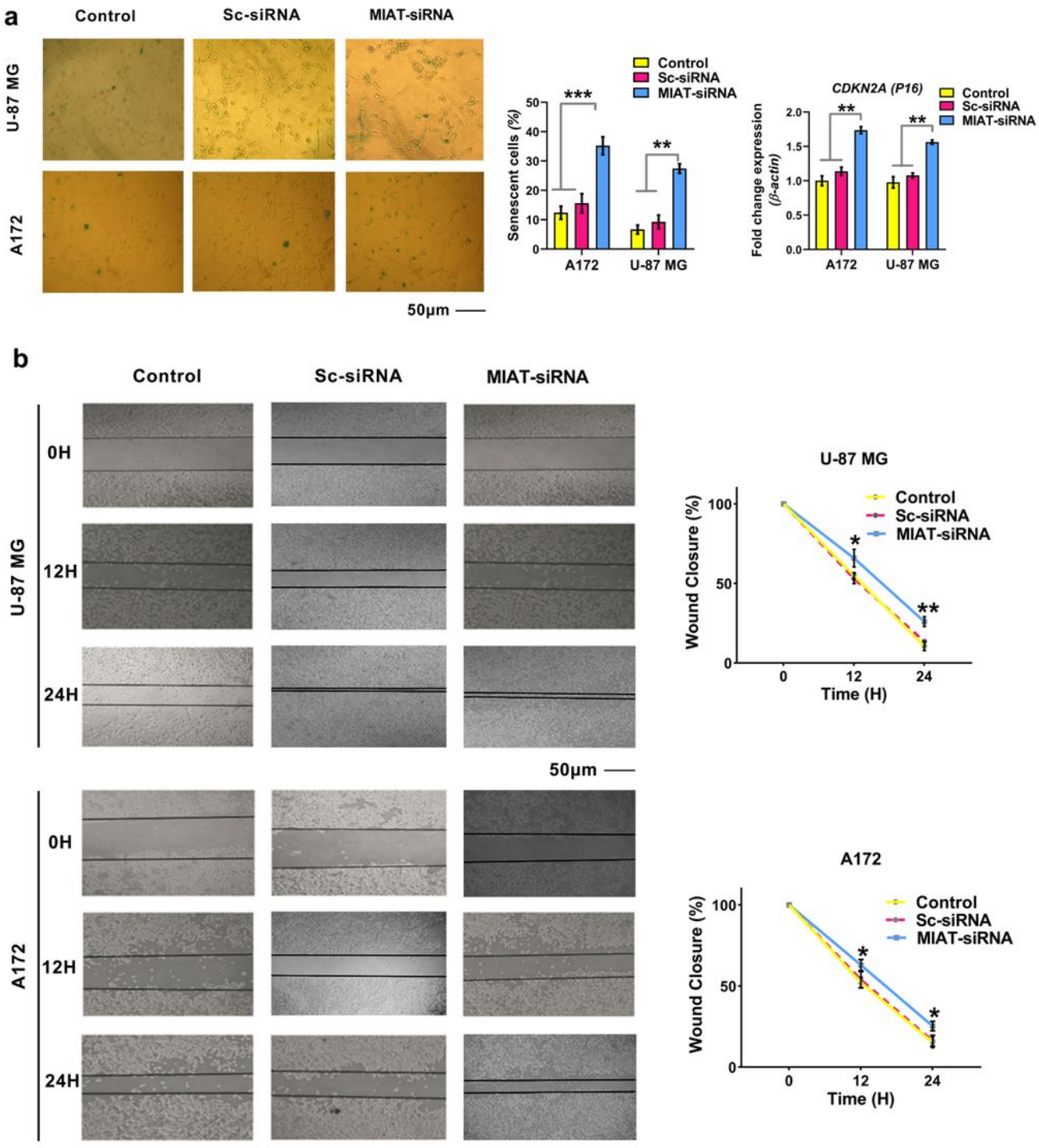

Figure 4

LncRNA MIAT knockdown increased the proportion of the senescent cells and limited the migration ability of glioma cancer cells. (a) The senescent cells proportion (positive cells for X-gal, stained green) significantly elevated when lncRNA MIAT was silenced in glioma cancer cells. (b) The migration of glioma cells reduced following LncRNA MIAT knockdown. Data are shown as mean ± SD based on triplicate independent replicates (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001)

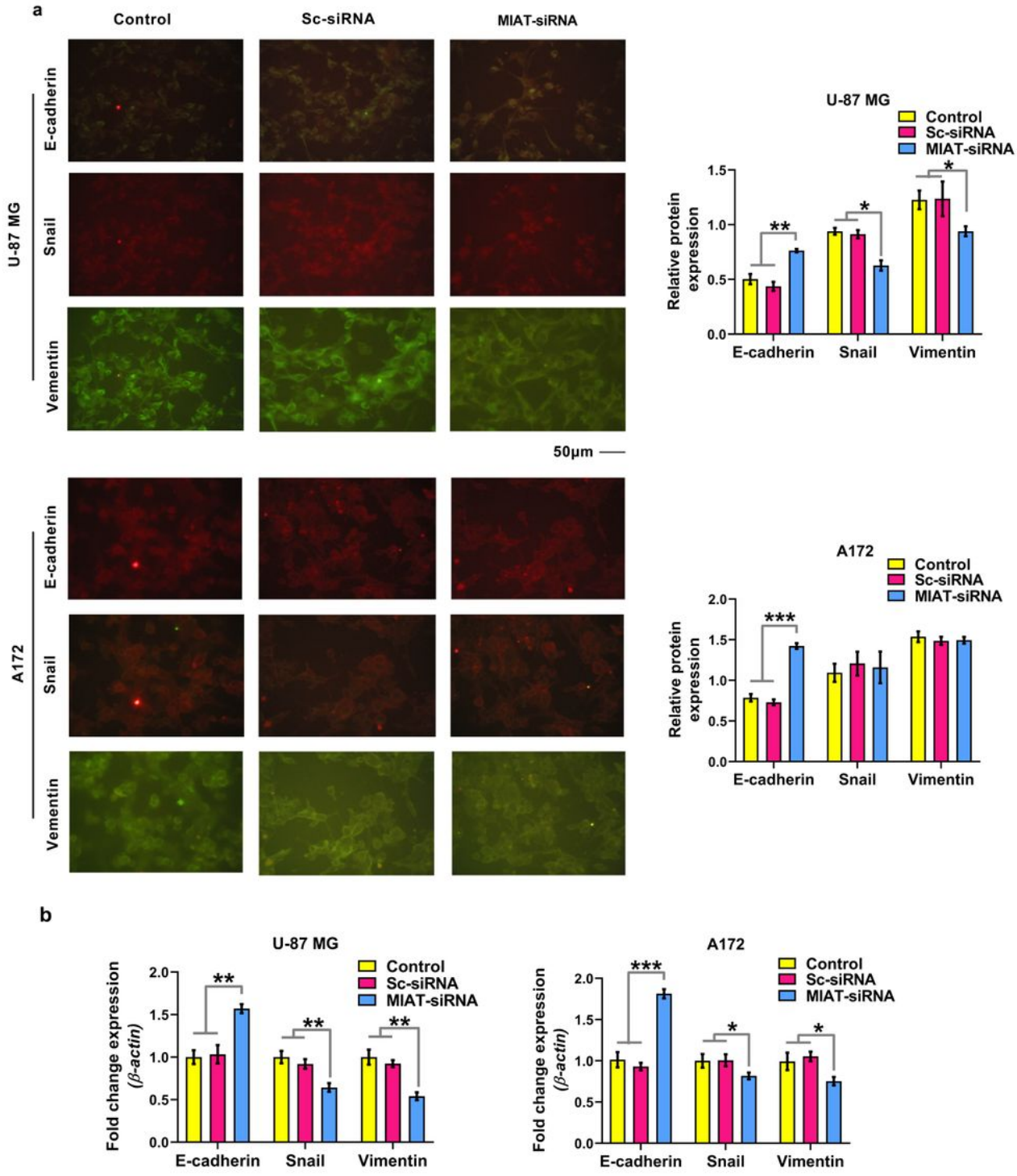

Figure 5

LncRNA MIAT knockdown decreased the EMT ability of the glioma cancer cells. (a-b)The expression of EMT markers at mRNA and protein levels demonstrated that lncRNA MIAT knockdown led to up-regulation of E-cadherin and down-regulation of Snail and Vimentin. Data are shown as mean ± SD based on triplicate independent replicates (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001)

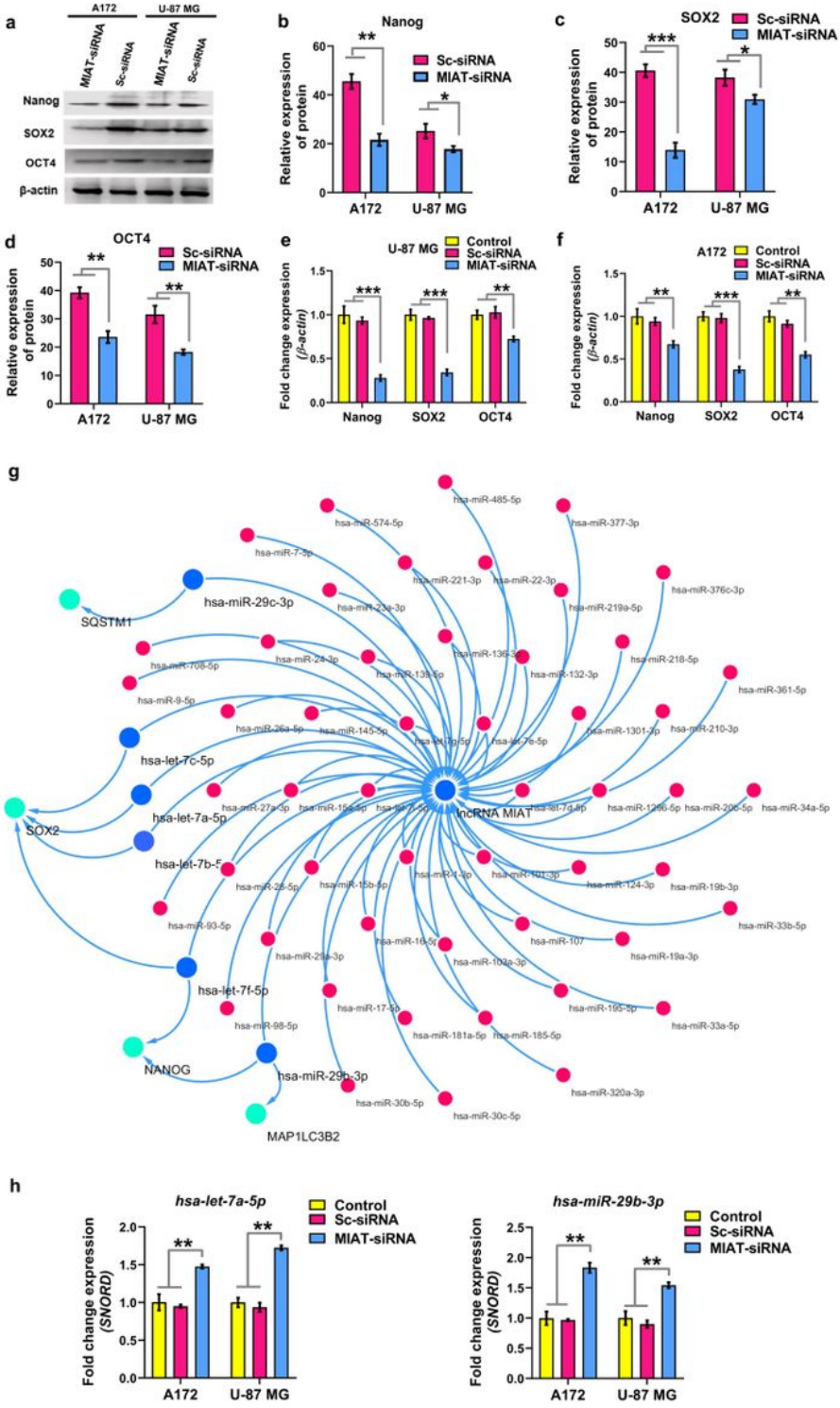

Figure 6

LncRNA MIAT knockdown reduced the expression of stemness factors and their associated miRNAs. (a-b-c-d) The expression of OCT4, SOX2 and Nanog significantly reduced after lncRNA MIAT down-regulation at the protein level. (e-f) The expression of OCT4, SOX2 and Nanog significantly also reduced after lncRNA MIAT down-regulation at the mRNA level. (g) All the miRNAs, binding to lncRNA MIAT, were extracted, and the targets of each miRNA were identified. Out-dated data was not depicted in the network to reduce the complexity. Central blue circle, lncRNA MIAT; Red circles, miRNAs; Arrows, mRNAs; Coral Blue, Target mRNAs of the selected miRNAs. (h) The expression level of let-7a-5p and miR-29b-3p dramatically increased following lncRNA MIAT knocking-down. Data are shown as mean ± SD based on triplicate independent replicates (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001)

Supplementary Files

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- SupplementaryFig.pdf