Abstract. Glioma is among the most common primary brain tumors and one of the most aggressive and lethal forms of human cancer. Long noncoding RNAs (lncRNAs) have demonstrated great importance in the development and progression of cancer. The present study aimed to investigate the role of the novel tumor suppressive lncRNA on Chromosome 8p12 (TSLNC8), in cell proliferation, metastasis and apoptosis in human glioma. It was initially reported that the relative transcript levels of TSLNC8 were significantly decreased in human glioma tissues and cultured glioma cells, as evidenced by RT-qPCR. Among clinical variables, the expression of TSLNC8 was negatively associated with tumor size, distant metastasis, and tumor, node and metastasis stage. MTT assay demonstrated that overexpression of TSLNC8 in glioma cell lines U25-MG and SWO38 decreased, whereas knockdown of TSLNC8 in glioma cells SHG-44 and BT325 increased the cell proliferative rate over 5 consecutive days. Additionally, cell metastasis was inhibited in U251 and SWO38 cells when cells were transfected with TSLNC8-expressing plasmid as observed via Transwell and wound-healing assays. Furthermore, cell apoptotic rate was upregulated in TSLNC8 plasmid-treated U251 and SWO38 cells, and inhibited by siRNA against TSLNC8 in SHG-44 and BT325 cells by cell apoptotic assay. The relative activities of caspase-3 and caspase-9 were increased by TSLNC8 overexpression and decreased by TSLNC depletion; however, the activity of caspase-8 remained unchanged. The results of the present study demonstrated the inhibitory effects of TSLNC8 in human glioma, which may contribute to advancement in the diagnosis and treatment of patients with glioma in clinic.

Introduction

Glioma is the most common malignancy of brain tumor, accounting for >32% of all brain tumors (1) and ~80% of malignancies in the brain (2). The overall median survival for patients with glioma is between 16 to 18 months (3,4). Notable advances in the therapeutic strategies of glioma have been made in the last decade, including neurosurgical approaches, chemotherapy and radiotherapy; however, the prognosis of glioma remains poor, mainly due to the fact that glioma cells are highly aggressive and capable of infiltrating adjacent normal brain tissue, leading to the failure of complete dissection of the tumor by surgery in the brain (5,6). Recurrence and resistance to chemotherapy are another two influential factors responsible for poor prognosis (7-9). Thus, developing novel strategies and investigating innovative therapeutic agents for patients diagnosed with glioma is imperative and urgently required.

Long noncoding RNAs (lncRNAs) are a class of RNAs that are non-protein coding and longer than 200 nucleotides in length (10-12). With the continuous advances of research approaches, lncRNAs are widely known and have recently undergone a rapid expansion in research and discovery. LncRNAs are closely associated with tumor development (13), particularly in the progression of glioma (14). For instance, lncRNA MEG63 contributes to cisplatin-induced apoptosis via the suppression of autophagy in human glioma (15). LncRNA CRNDE induces inflammation via the Toll-like receptor3-nuclear factor-xB-cytokine signaling pathway (16).

Tumor suppressive lncRNA on Chromosome 8p12 (TSLNC8) is a novel lncRNA, which is also named LINC00589 (17). TSLNC8 has been reported to be deleted and downregulated in human liver cancers. TSLNC8 serves its tumor suppressive role by physically interacting with transketolase and signal transducer and activator of transcription 3 (STAT3) and inactivation of the interleukin-6/STAT3 signaling pathway. In addition, TSLNC8 may inhibit the phosphorylation of STAT3, resulting in the low transcriptional activity of STAT3 in human liver cancer (17). Thus, TSLNC8 has been identified as a prognostic predictor for liver cancer patients in clinic; however, the role of TSLNC8 in human glioma requires further investigation.

In the present study, the transcript levels of TSLNC8 were observed to be significantly decreased in human glioma tissues.
compared with in noncancerous counterparts, and were negatively associated with tumor size, distant metastasis and tumor, node and metastasis (TNM) stage. Overexpression of TSLNC8 in glioma cells inhibited, while knockdown of TSLNC8 promoted cell proliferation and metastasis. TSLNC8 was also demonstrated to regulate cell apoptosis via the intrinsic pathway. The findings demonstrated the role of TSLNC8 in human glioma, which may contribute to the clinical diagnosis and treatment of the disease.

Materials and methods

Human samples. A total of 80 patients with diagnosed glioma (Male: Female, 59:21, age) were enrolled in the present study. For all patients, the dissected glioma tissues and their adjacent noncancerous tissues were collected from Tianjin First Center Hospital (Tianjin, China) during January 2014 to December 2016. All of the tissues were frozen in liquid nitrogen following dissection from patients during surgeries. Clinical data were also recorded for statistical analysis. Each patient provided informed consent to participate in the present study, which was approved by the Ethics Committee of Tianjin First Center Hospital.

Cell culture and transfection. Human glioma cell lines BE-2C and BT325 were purchased from the American Type Culture Collection (Manassas, VA, USA). Human glioma cell line SWO38 was a kind gift from Jinan University (Guangzhou, China) (18). Human glioma cell lines U251-MG, SHG-44 and CHG-5 were purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Science (Shanghai, China). Human normal neuronal cell line. Human normal astrocyte cells were purchased from Sciencell Research Laboratories, Inc. (San Diego, CA, USA; catalog no. 1830) and used as a normal control of glioma cell. All culture media (Dulbecco’s modified Eagle’s medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) were supplied with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.). Cells were maintained at 37˚C in a humidified atmosphere containing 5% CO₂. Small interfering (si)RNA against TSLNC8 was designed and synthesized by Shanghai Shenggang Biology Engineering Technology Service, Ltd., (Shanghai, China) with the sequence of 5′-GCA CAT GAA-3′ and the control siRNA sequence was 5'-GATTTTATTGAGTATACATG-3'. GAPDH forward, 5'-GGGACATCGCAAGGC-3' and reverse, 5'-AAAGGGTTGTAACGCACTA-3'.

Cell proliferation analysis. Cell viability was determined via an MTT assay. A total of 1,000 U251-MG and SWO38 cells were transfected with TSLNC8-overexpressing plasmid and SHG-44 and BT325 cells were treated with siRNAs with or without TSLNC8 knockdown for 48 h, trypsinized and reseded in triplicate in 96-well plates at an initial density of 4,000 cells per well. Cell numbers were monitored for a total of 5 consecutive days. At each indicated time points (days 1, 2, 3, 4 and 5), cell cultures were incubated with 10 µl MTT solution (5 mg/ml, Beyotime Institute of Biotechnology, Haimen, China) per well for 2 h at room temperature. The absorbance was recorded at a wavelength of 570 nm. Cell viability was defined as the cell number ratio of experimental groups to control cells.

Transwell assay. For cell migration assays, U251-MG and SWO38 cells were transfected with TSLNC8-overexpressing plasmid for 48 h and then trypsinized and collected by low-speed centrifugation (1,000 x g, 4˚C for 5 min) with serum-free DMEM. A total of 1x10⁴ cells (~150 µl) were spread into the upper chamber. The lower chamber was filled with 600 µl DMEM supplemented with 10% FBS. Subsequently, the plate was incubated for 24 h at 37˚C in an incubator and the cells were allowed to grow freely. At 24 h post-seeding, the membrane was fixed with pre-cooled methanol at room temperature for 10 min and stained with crystal violet (1%) for 5 min at room temperature. Cell migration was assessed by counting the cells that had migrated through the membrane. A total of 5 random fields were selected and images captured under a Nikon light microscope (Nikon Corporation, Tokyo, Japan) at a magnification of x100. For cell invasion assays, the membrane was pre-coated with Matrigel (Corning Incorporated, Corning, NY, USA) for 6 h at a 37˚C incubator.

Wound-healing assay. U251-MG and SWO38 cells were transfected with TSLNC8-overexpressing plasmid for 48 h and were then cultured in DMEM in a 6-well culture plate at a density of 5x10⁵ cells/well overnight until 90% confluence was attained. The culture medium was replaced with serum-free DMEM. A line was scratched in the single cell layer using a
10 µl pipette tip and the cells were then washed with PBS three times. Following incubation at 37˚C for 24 h, images of the migrated cells were observed and images were captured using a Nikon light microscope (magnification, x200).

**Cell apoptosis.** The Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) assay was performed according to the manufacturer’s protocol (Invitrogen; Thermo Fisher Scientific, Inc.). Briefly, a total of 10,000 U251-MG and SWO38 cells were plated into 6-well plates and transfected with TSLNC8-overexpressing plasmid for 48 h; SHG-44 and BT325 cells were transfected with control or specific siRNA against TSLNC8. Subsequently, cells were washed with pre-cooled PBS, trypsinized, and re-suspended in 100 µl of binding buffer with 2.5 µl FITC-conjugated Annexin V and 1 µl PI (100 µg/ml). Cells were then incubated at room temperature for 15 min in darkness. A total of ≥10,000 cells were collected and analyzed.

**Relative caspase activities.** The activities of caspase-3, -8 and -9 were determined using the caspase activity kits (Beyotime Institute of Biotechnology, Nantong, China) according to the manufacturer’s protocols. Briefly, U251-MG and SWO38 cells were plated into 6-well plates and transfected with TSLNC8-overexpressing plasmid for 48 h; SHG-44 and BT325 cells were transfected with control or specific siRNA against TSLNC8. Subsequently, cell lysates were collected by low speed centrifugation (1,000 x g, for 5 min at 4˚C). A total of 10 µl protein from each sample were added into 96-well plates and mixed with an aliquot of 80 µl reaction buffer supplied with caspase substrates (2 mM). Following incubation at 37˚C for 4 h, caspase activities were determined with a Tecan reader (Tecan Group, Ltd, Mannedorf, Switzerland) at an absorbance of 450 nm.

**Statistical analysis.** GraphPad Prism version 5.0 (GraphPad Software, La Jolla, CA, USA) software was used for statistical analysis. Data are presented as the mean ± standard deviation. The two-tailed Student's t-test was used to compare means of two groups, while one-way analysis of variance was used for comparisons among multiple groups (≥3 groups), followed by a Least Significant Difference post hoc test. The Spearman correlation analysis was used to evaluate the experimental data in Table I. P<0.05 was considered to indicate a statistically significant difference. Each experiment was repeated at least three times.

**Results**

*LncRNA TSLNC8 is downregulated in human glioma in vivo and in vitro.** First, the relative transcript levels of TSLNC8 in 80 glioma patients were analyzed in the present study. As
presented in Fig. 1A, the expression of TSLNC8 was significantly decreased in 95% cases of glioma patients (76 cases) compared with in adjacent noncancerous tissues (P<0.01). Of note, TSLNC8 transcript levels higher than the median level were characterized as high TSLNC8 expression (n=37); transcript levels lower than the median level were characterized as low TSLNC8 expression (n=43). The clinical data were also analyzed and presented in Table 1, which indicated that the relative transcript levels of TSLNC8 were negatively associated with tumor size (P=0.001), distant metastasis (P=0.004) and TNM stage (P=0.003), and not associated with age (P=0.2445), sex (P=0.960) and lymph node metastasis (P=0.155). Then, five glioma cell lines, including U251-MG, SHG-44, BT325, SWO38, CHG-5 and normal astrocyte cell line used as a control were analyzed. RT-qPCR analysis revealed that all of the five glioma cell lines exhibited significantly lower transcript levels of TSLNC8 compared with in the control astrocyte cells (Fig. 1B), of which U251-MG and SWO38 demonstrated the lowest expression levels of TSLNC8. Additionally, U251-MG and SWO38 cells exhibited the highest potential to migrate; SHG-44 and BT325 revealed the highest expression of TSLNC8 of the 7 glioma cell lines and the migration capacities of these cell lines were the lowest (Fig. 1B). Therefore, U251-MG and SWO38 were selected for the knockdown experiments and SHG-44 and BT325 were used for overexpression studies. All of these data showed that the transcript level of TSLNC8 was decreased in human glioma in vivo and in vitro.

Transcript levels of TSLNC8 are negatively associated with cell proliferation rate in human glioma. To investigate the role of TSLNC8 in human glioma, TSLNC8 was overexpressed in U251-MG and SWO38 cells and downregulated in SHG-44 and BT325 cells using an overexpression plasmid or siRNAs, respectively. When U251-MG and SWO38 cells were transfected with TSLNC8-overexpression plasmid, the transcript levels of TSLNC8 were significantly increased by 4.5- and 4-fold, respectively (Fig. 2A). In addition, the expression levels of TSLNC8 in SHG-44 and BT325 cells were significantly decreased by >50% of the original levels upon transfection with siTSLNC8 (Fig. 2B). Subsequently, the proliferation rate of the 4 glioma cell lines was analyzed. No significant alterations in the first 2 days in experimental cells were observed; however, cell proliferation decreased by 17% on day 4 and 19% on day 5 of U251 cells transfected with TSLNC8-overexpressing plasmid (Fig. 2C). Furthermore, SWO38 cell proliferation rate decreased on days 4 and 5 with TSLNC8 overexpression (Fig. 2D); the cell proliferation rate increased on days 4 and 5 in SHG-44 and BT325 cells transfected with siRNA against TSLNC8 (Fig. 2E and F, respectively). These results suggested that TSLNC8 may suppress cell proliferation in human glioma cells.

Transcript levels of TSLNC8 are negatively associated with cell metastasis in human glioma. As well as cell proliferation rate, the effects of TSLNC8 on cell metastasis were investigated. To this end, TSLNC8-overexpressing plasmid was transfected into U251-MG and SWO38 cells for 48 h. Transwell assays revealed that >400 U251 and SWO38 cells were observed to migrate via the membrane; however, only ~200 cells were counted on the lower side of the membrane upon transfection with TSLNC8-expressing plasmid (Fig. 3A and B). Additionally, cell invasion was also inhibited by TSLNC8 overexpression in both U251-MG and SWO38 cells (Fig. 3A and C). Subsequently, wound-healing assays were also performed in U251-MG and SWO38 cells. As presented in Fig. 3D and E, the wound closure ability of U251-MG cells was inhibited by >50% when cells were treated with TSLNC8-overexpression plasmid. Furthermore, the wound closure ability of SWO38 cells was also inhibited by 50% upon TSLNC8 overexpression (Fig. 3D and F). These data suggested that TSLNC8 suppressed cell metastasis in human glioma.

Transcript levels of TSLNC8 are positively associated with cell apoptosis in human glioma. Finally, cell apoptosis was assessed in glioma cells. As presented in Fig. 4A, cell apoptosis was increased by 2.7-fold in U251-MG cells and 2.5-fold in SWO38 cells of the TSLNC8 treated groups compared with in the control cells; the cell apoptotic rate of SHG-44 and BT325 cells were significantly decreased by >50% upon siTSLNC8 stimulation (Fig. 4B). Subsequently, the relative caspase activities were analyzed. Treatment with TSLNC8-expressing plasmid of U251-MG and SWO38 cells revealed that the expression of TSLNC8 was significantly increased >50% upon siTSLNC8 stimulation (Fig. 4B). Therefore, U251-MG and SWO38 were selected for the knockdown experiments and SHG-44 and BT325 were used for overexpression studies. All of these data showed that the transcript level of TSLNC8 was decreased in human glioma in vivo and in vitro.

Figure 1. Long noncoding RNA TSLNC8 is downregulated in human glioma in vivo and in vitro. (A) RT-qPCR analysis were performed in tumor tissues and their adjacent noncancerous tissues from 80 clinical glioma patients. *P<0.01. (B) RT-qPCR analysis were performed in glioma cell lines (U251-MG, SHG-44, BT325, SWO38, and CHG-5) and a normal astrocyte cell line, which served as the control. *P<0.05 vs. control. RT-qPCR, reverse transcription-quantitative polymerase chain reaction; TSLNC8, tumor suppressive lncRNA on Chromosome 8p12.
increased, while stimulation of siTSLNC8 in SHG-44 and BT325 cells decreased the activities of caspase-3, respectively (Fig. 4C and D). Interestingly, the activity of caspase-8 remained stable upon transfection with TSLNC8-expressing plasmid or siTSLNC8 in glioma cells (Fig. 4E and F). The relative activities of caspase-9 were also significantly altered, following similar trends to those exhibited by caspase-3, in the different glioma cell lines (Fig. 4G and H). As caspase-3 and -9 are key factors involved in the intrinsic apoptosis pathway and caspase-8 serves significant roles in the extrinsic apoptotic pathway (20), the findings of the present study suggested that TSLNC8 may promote cell apoptosis via the intrinsic pathway in human glioma.

Discussion

Deletion in the short arm of chromosome 8 is among the most common genetic events in a variety of cancers, and deletions include Rho guanosine 5'-triphosphate-ase activating protein (8p22) (21), leucine zipper tumor suppress 1 (8p21) (22) and tumor necrosis factor superfamily member 10c (8p21) (23). The present study demonstrated that a novel IncRNA TSLNC8, also located at 8p12, may serve a significant role in human glioma, which was consistent with the former findings of Zhang et al (17). The results of the present study suggested that TSLNC8 was downregulated in human glioma, which was associated with increased cell proliferative rate and cell...
metastasis, as well as the decreased cell apoptotic capacity in human glioma cell lines; TSLNC8 may be a potential therapeutic target for cancer patients in clinic.

Cell proliferation and cell metastasis are two primary manifestations in the majority of malignancies (24-25). Initiation of metastasis requires invasion, which was examined in the present study via Transwell and wound-healing assays. Invasion is enabled by epithelial-mesenchymal transition (EMT) (26,27). Cells from primary tumors lose cell-cell adhesion mediated by E-cadherin repression and break through the basement membrane with increased invasive capacity, thus enter the bloodstream via intravasation (28). At novel metastatic sites, tumor cells undergo mesenchymal-epithelial transition by overexpressing N-cadherin (28); the protein levels of E-cadherin and N-cadherin require further investigation in future studies, as well as the detailed mechanism of the regulatory effects of TSLNC8 on human glioma.

Figure 3. Transcript levels of TSLNC8 are negatively associated with cell metastasis in human glioma. (A) Representative images of Transwell assays in U251-MG and SWO38 cells in the presence and absence of TSLNC8 overexpression. Magnification, x200. (B) Quantification of cell migration assays of U251-MG and SWO38 cells in the presence and absence of TSLNC8 overexpression. (C) Quantification of cell invasion assays of U251-MG and SWO38 cells in the presence and absence of TSLNC8 overexpression. (D) Representative images of wound-healing assays in U251-MG and SWO38 cells in the presence and absence of TSLNC8 overexpression. (E) Quantification of wound healing assays of U251-MG cells in the presence and absence of TSLNC8 overexpression. (F) Quantification of wound healing assays of SWO38 cells in the presence and absence of TSLNC8 overexpression. *P<0.05 vs. Control. TSLNC8, tumor suppressive lncRNA on Chromosome 8p12.
Numerous factors are involved in EMT, including programmed death-ligand 1, twist-related protein 1 and 2, and transforming growth factor β1 (TGFβ1). TGFβ1 may promote tumor invasion and evasion of immune surveillance at the advanced stage of malignancies (29). EMT is favored and apoptosis is suppressed when TGFβ1 acts on activated Ras-expressing mammary epithelial cells, which may be reversed by the inducers of epithelial differentiation (30). As the inhibition of cell apoptosis is a good basis for tumor cell growth, the role of TSLNC8 in cell apoptosis was analyzed in the present study. TSLNC8 was observed to promote cell apoptosis by increasing the activities of caspase-3 and caspase-9 in the intrinsic pathway in the present study. The mitochondria-mediated intrinsic pathway is dependent on the release of cytochrome c, which leads to the caspase-9-dependent activation of caspase-3 (31). Conversely,
the death receptor-induced extrinsic pathway signals in a caspase-8 dependent manner (31). The overexpression of TSLNC8 promoted, whereas knockdown of TSLNC8 inhibited the activities of caspase-3 and caspase-9, but not caspase-8 in the present study. However, the detailed mechanisms of the promoting effects of TSLNC8 on cell apoptosis in human glioma require further investigation in the future.

In conclusion, the present study revealed the importance of a novel IncRNA, TSLNC8, in human glioma. TSLNC8 was demonstrated to inhibit cell proliferation and metastasis, and promote cell apoptosis in glioma cells. These findings indicated that TSLNC8 may serve as a potential therapeutic target for the diagnosis and treatment of glioma in clinic.

Acknowledgements

Not applicable.

Funding

Not applicable.

Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Authors’ contributions

DC performed the experiments. XY designed the study, analyzed the data and wrote the manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Tianjin First Center Hospital. Each patient provided informed consent to participate.

Patient consent for publication

Written informed consent was obtained.

Competing interests

The authors declare that they have no competing interests.

References


This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0) License.