Long non-coding RNA LINC00525 regulates 
the proliferation and epithelial to 
mesenchymal transition of human glioma cells by sponging miR-338-3p

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Abstract

Long non-coding RNA (LncRNA) LINC00525 has been shown to be upregulated in several human cancers and 
deduced to possess cancer regulatory role. The regulation of molecular mechanics of human glioma by IncRNA- 
LINC00525 through microRNA sponging in glioma is elusive. The IncRNA-LINC00525 showed significant (P < 0.05) 
upregulation in glioma cancer cells. The upregulation of IncRNA-LINC00525 was up to 6.6-fold in glioma cells relative 
to the normal cells. Knockdown of IncRNA-LINC00525 significantly declined the proliferation of the glioma cancer 
cells. Additionally, the colony formation was inhibited by around 60% in glioma cells. The wound healing and tran-
swell assays revealed significant (P < 0.05) inhibition of migration and invasion potential under IncRNA-LINC00525 
knockdown. The western blotting study of biomarkers of epithelial to mesenchymal transition (EMT) revealed that 
IncRNA-LINC00525 gene silencing reduced the expression of mesenchymal molecular markers but increased the 
protein levels of epithelial markers. miR-338-3p was predicted to be interacting with IncRNA-LINC00525 in glioma 
and was shown to mediated the regulatory role of IncRNA-LINC00525. Taken together, the results of present study are 
supportive of the prognostic applicability of IncRNA-LINC00525 against human glioma together with its therapeutic 
potential against the said malignancy.

Keywords: Glioma, Long non-coding RNA, microRNA, Proliferation, Migration, Invasion, Glioblastoma

Introduction

The human gliomas are considered as the most prevalent types of human central nervous system and are 
broadly classified into the three broad pathological categories, which include oligodendrogliomas, astrocytomas 
and ependymomas (Goodenberger and Jenkins 2012). Approximately, five new cases of glioma per 1 lakh of 
human population are diagnosed annually. Out of the total cases diagnosed, more than 50% cases are found 
to be manifesting the grade IV astrocytomas referred as glioblastomas (Schwartzbaum et al. 2006). The glioblas-
toma is highly aggressive and patients affected with this disorder have 5 year survival rate of not more than year 
and a half (Bush et al. 2017). Moreover, it is seen to more commonly affect the male population with 1.5 times 
higher prevalence rates (Nizamutdinov et al. 2018). The glioblastoma is currently being treated through max-
imal safe surgical resection combined with the controlled application of radio- and chemotherapeutic treatments 
(Bianco et al. 2017). Despite such clinical treatment measures, the over-all survival rate of glioblastoma has 
not enhanced much. The inefficiency of the treatment strategy is attributed to difficulty of disease prognosis at 
early stages (Bush et al. 2017). The researchers are thus in continuous exploration of the effective prognostic
and treatment tactics for obtaining better clinical outcomes against the malignant type of human gliomas. The research approaches seen with promising results towards this direction include the discovery of specific diagnostic and therapeutic targets to be utilized for the management of human glioma. The non-coding RNAs consisting of long non-coding RNAs (lncRNAs) and microRNAs (miRs) are being extensively studied for their cancer regulatory effects in humans (Slaby et al. 2017). The research findings have clearly indicated that lncRNAs and miRs regulate the molecular mechanics of growth and proliferation of cancer including the human gliomas and there is growing support for these RNA bio-molecules to serve as the prognostic and therapeutic agents against the glioma (Peng et al. 2018; Shi et al. 2017). The dysregulation of IncRNA-LINC00525 and miR-338-3p has been implicated to be associated with several human cancers (Wang et al. 2017; Zhang et al. 2017). However, the regulatory interactional interplay of IncRNA-LINC00525 and miR-338-3p has not been worked out in glioma.

We, in the present study, found that the glioma cancer cell lines exhibited significantly higher transcript levels of IncRNA-LINC00525 and the experimental silencing of IncRNA-LINC00525 not only reduced the growth of the cancer cells but also rendered them to migrate and invade at significantly lower rates, in vitro. Interestingly, it was deduced that IncRNA-LINC00525 positively regulates the epithelial–mesenchymal transition (EMT) of glioma cells and its knockdown markedly enhanced the expression of epithelial molecular marker proteins but declined the protein levels of mesenchymal biomarkers. The IncRNA-LINC00525 was found to be sponging the expression of miR-338-5p as the latter was confirmed to interact with IncRNA-LINC00525 in sequence directed manner. The anti-proliferative effects of IncRNA-LINC00525 knockdown were shown to be operated through transcript level enhancement of tumor suppressor miRNA-338-3p. The study thus deduced that IncRNA-LINC00525 might emerge as crucial prognostic marker against human glioma and showed the therapeutic value of IncRNA-LINC00525 targeting through miR-338-3p.

Materials and methods

Growth and maintenance of cell lines and their transfection

The American Type Collection Center (ATCC) provided the glioma cancer cell lines (U87, MO59K, U118, Hs683 and LN-18) as well as the normal astrocyte cell line. Dulbecco’s Modified Eagle’s medium (DMEM) (Sigma-Aldrich, St. Louis, Missouri, USA) supplemented with high concentration glucose was used for culturing of all the cell lines using 37 °C temperature and 5% CO₂ concentration conditions which were maintained using humidified growth incubator.

Transfection

The transfection of glioma cell lines was performed with the help of Lipofectamine 2000 (Thermo Fisher Scientific) reagent as per the manufacturer’s protocol. Various constructs used for transfection of cancer cells were ordered from Guangzhou RiboBio Co. Ltd (Guangzhou, China).

Synthesis of complementary DNA (cDNA) and expression analysis

Prior to the real time polymerase chain reaction (RT-PCR) study, the extraction of total RNA from the normal astrocyte and cancer cell lines and its subsequent reverse transcription to cDNA was performed with the help of TRIzol reagent (Thermo Fisher Scientific) and HiScript II qRT SuperMix kit (Vazyme, China), respectively. The cDNA was used as template and SYBR Green PCR master mix (Thermo Fisher Scientific) was used for performing the RT-PCR reaction. The PCR was run on the QuantStudio 5.0 PCR system (Applied Biosystems). The 2−ΔΔCt method based calculations were performed to examine the relative gene expression levels. For the normalization of the RT-PCR expression analysis, the human GADPH gene was used as an internal control.

Cell viability assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (sigma-Aldrich) assay was used to determine the viability of transfected glioma cells. Briefly, 2 × 10⁴ cells were added per well of a 96-well plate and cultured in DMEM medium supplemented with high glucose concentration at 37 °C for 0 h, 12 h, 24 h, 36 h, 48 h, 72 h or 96 h. The MTT solution at final concentration of 0.5% was added to each well of the plate and 4 h incubation was given at 37 °C. The dissolution of formazan crystals was made by the addition of dimethyl sulfoxide (DMSO). Finally, the absorbance values were measured at 570 nm using ELx-800 Universal Microplate Reader. At least three replicas were kept for each experimental sample.

Colony formation assay

The clonogenic colony forming assay was determined to assess the relative colony formation by the transfected glioma cells, in vitro. Here, about 2000 transfected cells were added to each well of the 6-well plate and cell culturing was performed for a period of 2 weeks at 37 °C in humidified incubator. After, 2 weeks the colonies were visualized by light microscope after their washing with PBS, fixation by ethanol and staining with 0.1% crystal
violet solution. The relative percentage of the colonies formed was calculated using five random microscopic fields per sample.

**Wound-heal assay**
The migration of the transfected glioma cells was determined through the wound-heal assay. Briefly, the transfected cells were cultured in 6-well plates at $2 \times 10^5$ cells per well in DMEM at 37 °C until a regular cell surface was obtained. The surface was then carefully scratched to carve a linear wound with the tip of a 10 μl micropipette which was photographed under the light microscope. The 37 °C incubation was continued for 24 h following which the wound was again visualized using microscope and its width was compared with the initial wound width. Three replicates were used for carrying out the experiment.

**Transwell chamber assays**
The transwell assay was used for the estimation of invasion of the transfected glioma cancer cells through the transwell membrane coated with Matrigel. The transfected cells were inoculated at cellular density of $1 \times 10^4$–$1 \times 10^5$ cells per well in the upper chamber of transwell while the lower chamber was filled with DMEM medium only. The plate was incubated at 37 °C for 48 h. The cells which invaded the lower chamber were fixed with 70% ethanol, crystal violet (0.1%) staining was performed. The cells were then examined under light microscope. The cell counting was done and the relative percentage of the cell invasion was estimated using multiple random microscopic fields. The experiments were performed with three replicates.

**In silico analysis**
To specifically identify the microRNA which targets lncRNA-LINC00525 in glioma, the in silico analysis was performed using StarBase v2.0 (https://starbase.sysu.edu.cn/starbase2/index.php) online database. The online bioinformatics was further used to identify the specific lncRNA-LINC00525 sequence used for microRNA binding.

**Western blotting**
Total cellular proteins from the transfected cells were extracted with the help of RIPA lysis and extraction buffer (Thermo Fisher Scientific) according to the manufacturer's protocol. The protein quantification was done using Bio-Rad protein assay kit. The separation of the protein extracts was performed using 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE). Afterwards, the proteins were electrophoretically transferred to the nitrocellulose membranes which were exposed to specific antibodies for 12 h at 4 °C in dark. This was followed by the incubation of the membranes to peroxidase-linked secondary antibodies 2 h at room temperature. The protein bands were visualized using the enhanced chemiluminescence. Human β-actin protein was recognized as the internal control in the protein blotting studies.

**Statistical analysis**
The experiments were performed in triplicate and expressed as mean ± SD. Student's t-test (for comparison between two samples) and one way analysis of variance followed by Tukey’s test (for comparison between more than two samples) were used for statistical analysis using GraphPad Prism software (version 7; GraphPad Software, Inc., La Jolla, CA, USA. P < 0.05 was considered to indicate a statistically significant difference.

**Results**

**Glioma cells exhibit transcript upregulation of lncRNA-LINC00525**
To know about the expression of lncRNA-LINC00525 in human glioma, the RT-PCR was performed for analyzing its transcript abundance in glioma cell lines (U87, MO59K, U118, Hs683 and LN-18) in comparison to the normal astrocyte cell line. It was seen that all the cancer cell lines exhibited significantly (P < 0.05) higher lncRNA-LINC00525 expression than the normal astrocytes (Fig. 1a). The upregulation of lncRNA-LINC00525 was up to 6.6 folds in glioma cells. Highest expression levels were noted from the U87 and U187 cell lines and thus these were taken for the further characterization of role lncRNA-LINC00525 in glioma. The results are thus indicative of dysregulation of lncRNA-LINC00525 in glioma suggesting its probable molecular regulatory role in the malignancy.

**LncRNA-LINC00525 knockdown inhibited the glioma cell growth**
The U87 and U187 cell lines were transfected with si-LINC00525 construct to perform the experimental gene silencing of lncRNA-LINC00525 in these cell lines. The RT-PCR expression study of lncRNA-LINC00525 showed that the si-LINC00525 transfected cells exhibited significant transcriptional repression (5.3-fold) of lncRNA-LINC00525 in comparison to the respective normal control cell lines transfected with si-NC (Fig. 1b). The proliferation of cancer cells under lncRNA-LINC00525 knockdown was shown to be significantly (P < 0.05) lower in comparison to the control cells, as deduced by MTT assay (Fig. 1c).
LncRNA-LINC00525 knockdown inhibited the colony formation of glioma cells

The effects of LncRNA-LINC00525 was assessed by colony formation assay. The results showed that U118 and U87 glioma cell colonies decreased significantly upon RNA-LINC00525 silencing (Fig. 2a, b). The colonies were inhibited by almost 60% in both U118 and U87 glioma cells. These results suggest that LncRNA-LINC00525 regulates the growth of glioma cancer cells and its intentional silencing might serve a therapeutic role against the human glioma.

LncRNA-LINC00525 silencing suppressed glioma cell migration and invasion

The metastasis of cancer cells is one of the crucial aspects of aggressiveness of a particular human cancer (Steeg 2016). Thus, scientists are continuously looking for the measures to contain the motility of cancer cells. The assessment of the migration of glioma cells showed that the cancer cells showed significantly lower migration under LncRNA-LINC00525 knockdown (Fig. 3). The invasion of the glioma cells was also seen to significantly fall under LncRNA-LINC00525 knockdown (Fig. 4). Together the results show that LncRNA-LINC00525 might be positively regulating the metastasis of glioma cells and thus its molecular targeting may assist is restricting the glioma spread.

The regulation of epithelial to mesenchymal transition of glioma cells by LncRNA-LINC00525

The study of the protein expressions of the molecular markers of epithelial–mesenchymal transition (EMT) showed that the knockdown of LncRNA-LIN00525 led to the enhancement in the protein levels of the epithelial molecular markers (E-cadherin and α-catenin) while it significantly reduced the expression of the mesenchymal protein markers (fibronectin and vimentin) (Fig. 5). Similar trends were observed for U87 and U118 cell lines. The results that show that LncRNA-LINC00525 might be regulating the EMT of glioma cells further revealing its therapeutic against the human glioma.
LncRNA-LINC00525 exercised glioma regulatory role through miR-338-3p

To specifically predict the microRNA targeting lncRNA-LINC00525 in glioma, in silico analysis was used. The bioinformatics showed that miR-338-3p is interacting with lncRNA-LINC00525 in a sequence specific manner in glioma (Fig. 6a). The prediction was supported by the RT-PCR study of miR-338-3p in glioma cell lines. The cancer cell lines exhibited significantly lower miR-338-3p expression corresponding to higher lncRNA-LINC00525 levels in comparison to the normal astrocytes (Fig. 6b). miR-338-3p was over-expressed in U87 and U118 cell lines (Fig. 7a). The miR-338-3p over-expression further reduced the proliferation of glioma cancer cells, in vitro (Fig. 7b). The results thus suggest that lncRNA-LINC00525 might be regulating the mechanics of growth and proliferation of glioma cancer cells via its sponging of miR-338-3p.

Discussion

Gliomas are the most aggressive types of human cancers mainly affecting central nervous system. The gliomas constitute 50% of all the primary malignant tumors of central nervous system and exhibit high mortality and incidence (Vallée et al. 2017). The grade four glioma patients have an average survival period of just 16 months (Yu et al. 2017). The human glioma is currently been treated though the application of careful surgical resection combined with radiotherapy/radiotherapy and chemotherapy, particularly the administration of temozolomide (Bianco et al. 2017). However, the over-all survival rate of glioma is still very low and the glioma patients hardly survive for a year and half (Bush et al. 2017). Thus, the currently attempted clinical are not much admirable and it is therefore needed to look for the alternative therapeutic procedures which may prove more effective and to lessen the burden of this malignancy. Originally described as the noise of eukaryotic genome, the long-coding RNAs (lncRNAs) have emerged as the extensively studied bio-molecules for their prognostic role and therapeutic value against the human cancer (Lin and Yang 2018). The human cancers have been shown to express aberrant transcript levels of lncRNAs. Numbers of lncRNAs display either significant higher or lower expression in human gliomas and have been suggested to serve in the disease prognosis (Rao et al. 2017; Lin et al. 2019; Wang et al. 2019). In the...
Fig. 3 lncRNA-LINC00525 knockdown restricts the glioma cell migration. Analysis of migration U87 and U118 cancer cells transfected with si-NC or si-LINC00525 by wound-heal assay. The experiments were performed in triplicate and expressed as mean ± SD (P < 0.05).

Fig. 4 lncRNA-LINC00525 knockdown restricts the glioma cell invasion. Analysis of invasion U87 and U118 cancer cells transfected with si-NC or si-LINC00525 by transwell assay. The experiments were performed in triplicate and expressed as mean ± SD (*P < 0.05).
In the present study, the expression of lncRNA-LINC00525 was significantly higher in the glioma cancer cells than the normal astrocyte cell line and this observation fits well with the already established findings making it suitable to serve as the prognostic biomarker in diagnosing human glioma (Peng and Zheng 2019). The silencing of lncRNA-LINC00525 declined the growth of glioma cells and glioma cells proliferated at remarkably lower rates. The basis of such anti-proliferative effects of lncRNA-LINC00525 was shown to be its regulatory control on the cell apoptosis (Zheng and Peng 2019). Interestingly, the results of the present research study indicated that lncRNA-LINC00525 has pro-metastatic regulatory role in human glioma. The lncRNA-LINC00525 knockdown in glioma cells negatively affected their migration and invasion potential. The reason behind such anti-motility effect might be decline of cell viability by the transcriptional silencing of lncRNA-LINC00525 together with its microRNA sponging action in glioma. The suppression of migration and invasion by lncRNA-LINC00525 has been also reported for the non-small lung cancer cells (Yang et al. 2020). Moreover, the key finding of the present study was the elucidation of regulation of epithelial–mesenchymal transition (EMT) of glioma cells by lncRNA-LINC00525. EMT is responsible for the tumor-ogenesis of human cancer and known to fundamentally mediate the cancer cell metastasis (Kiesslich et al. 2013). During EMT, the cells lose their adhesion with the basement membrane and undergo several morphological transformations with the alterations of expression of proteins which are used as the bio-markers for this biological cellular transition (Moreno-Bueno et al. 2009). The silencing of lncRNA-LINC00525 increased the expression of epithelial markers (E-cadherin and α-catenin) while it was seen to decrease the expression of mesenchymal marker proteins (vimentin and fibronectin) (Sethi et al. 2011). At the molecular level, LINC00525 was shown to mediate its regulatory effects through the sponging of miR-338-3p which has been also confirmed by the previous studies in non-small lung cancer (Yang et al. 2020). Summing up, the study signifies the prognostic application and therapeutic potential of lncRNA-LINC00525 against the human glioma and emphasized on the importance of molecular regulatory axis, miR-338-3p/lncRNA-LINC0025 for its therapeutic targeting against human glioma.

Taken together, the study showed glioma cells expressed significantly higher transcripts of lncRNA-00525. This transcriptional dysregulation together with the findings that lncRNA-LINC00525 through sponging of miR-338-3p regulates the growth and metastasis of human glioma cancer cells and is exerting a regulatory control over their EMT revealed that lncRNA-00525 might serve as potential prognostic marker against the human glioma and the miR-338-3p/lncRNA-LINC0025 molecular axis might be employed as a vital therapeutic target against human glioma in future.
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Authors’ contributions
YW, MW and YL designed the protocol of the study. YW, MW, FL and YL performed the experimental work and collect the data for presented study. MW and YL involve in the statistical analysis. MW and YL supervised the work and drafted the manuscript, although all author contributes for the preparation of manuscript. All authors read and approved the final manuscript.

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Fig. 7 The regulatory effects of lncRNA-LINC00525 are exerted though miR-338-3p in human glioma. a Expression analysis of miR-338-3p from U87 and U118 cancer cells transfected with miR-NC or miR-338 mimics, b expression analysis of lncRNA-LINC00525 from U87 and U118 cancer cells transfected with miR-NC or miR-338 mimics, c the estimation of proliferation of U87 and U118 cancer cells transfected with miR-NC or miR-338 mimics by MTT assay. The experiments were performed in triplicate and expressed as mean ± SD (*P < 0.05)
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