Long non-coding RNA HULC regulates growth and metastasis of human glioma cells via induction of apoptosis and inhibits cell migration and invasion

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Submitted: 13 August 2020
Accepted: 12 October 2020

Arch Med Sci
DOI: https://doi.org/10.5114/aoms.2020.100834
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Abstract

Introduction: The long non-coding RNA HULC has been shown to be involved in the development of several human cancers. The present study was undertaken to investigate the regulatory role of IncRNA-HULC in growth and metastasis of human glioma.

Material and methods: The gene expression of IncRNA-HULC was estimated from the clinical glioma tissues and cell lines using RT-PCR. The proliferation of transfected cancer cells was determined with the help of cell counting kit-8 (CCK8). DAPI staining and dual annexin V-FITC/PI staining procedures were used for inferring the apoptosis of transfected cancer cells. Scratch-heal and transwell chamber assays were employed for the determination of migration and invasion of transfected cells. The expression of proteins of interest was studied by western blotting technique.

Results: The results showed that IncRNA-HULC exhibits significantly ($p < 0.05$) higher expression in glioma tissues and cancer cells. The knockdown of IncRNA-HULC led to a marked decline in the proliferation of glioma cells through apoptotic induction which was accompanied by upregulation of Bax and downregulation of Bcl-2. Moreover, knockdown of IncRNA-HULC significantly ($p < 0.05$) suppressed the migration and invasion of cancer cells in vitro. The western blot analysis showed that IncRNA-HULC exerted its effects via modulation of the PI3K/AKT signaling pathway.

Conclusions: The study revealed the possibility of targeting the PI3K/AKT signaling pathway in glioma through transcriptional knockdown of IncRNA-HULC, which might be utilized for therapeutic purposes against human glioma.

Key words: glioma, long non-coding RNA, proliferation, apoptosis, transcriptional knockdown, migration, invasion.

Introduction

The gliomas include the most dominant form of endocrine tumors affecting the human brain and cause tremendous human mortality at the global level [1]. Histologically, the human gliomas are deemed to emerge from the neuro-epithelial tissue and are categorized based on clinical features and histo-pathological parameters [2]. Among the sub-types of glioma, the glioblastoma is considered to be the most lethal and an extremely aggressive tumor [3]. The average survival period of glioblastoma patients is less than 1 year after disease diagnosis [4]. Currently,
patients with glioma are treated with the combi-
natorial application of surgical and chemo and/or
radiotherapeutic procedures. This combined ther-
aputic approach has enhanced the survival rate
of glioma, but satisfactory clinical outcomes are
yet to be achieved. Therefore, it is crucial to un-
derstand the in-depth pathology of glioma at the
molecular level to develop more efficient therapeutic
approaches against this lethal malignancy. During
the recent era, research findings have broadened
our understanding and knowledge about the
cancer and have led to the identification of vital
regulatory players responsible for controlling the
growth and progression of human cancers. Such
studies also showed the potential of the long
non-coding RNAs (lncRNAs) to serve in the prog-
nosis of various cancerous disorders together
with their utility as potent anti-cancer therapeutic
biomolecules [5]. LncRNAs are described as RNA
molecules, ranging in size from 200 to more than
100 thousand nucleotides in length, which do not
code for proteins but function as transcriptional
regulators [6]. It has been reported that lncRNAs
assist in tissue homeostasis and regulate the
human cancer progression besides serving other
biological and physiological roles [7]. A good num-
ber of studies have shown that aberrant transcript
levels of lncRNAs profoundly affect the initiation
and progression of human gliomas [8, 9]. In this
regard, we tried to investigate the regulatory ef-
fects of lncRNA-HULC in controlling the growth
and spread of glioma by taking advantage of the
cell line system. The lncRNA highly up-regulated
in liver cancer (HULC) was identified as the ln-
cRNA most up-regulated expressed in hepatocel-
ular carcinoma in 2007 [10]. It is located at the
chromosomal section 6p24.3 and has a length of
about 500 nucleotides consisting of two exons. Lnc-
rNA-HULC has been suggested to play a critical
role in various human cancers. It is shown to modu-
late the HBx/STAT3/miR-539/APOBEC3B signal-
ing pathway to regulate HBV-related hepatocellular
carcinoma [11]. LncRNA-HULC down-regulates
miR-15a to promote growth, migration and inva-
sion of pancreatic cancer cells and acts as a prog-
nostic factor of pancreatic cancer [12, 13]. Howev-
er, its role in glioma is still not well known. Thus,
the present study was aimed at the exploration of
this together with determination of the molecular
mechanism mediating the regulatory role of lnc-
rNA-HULC in human glioma. The results revealed
lncRNA-HULC as an important regulator of growth
and metastasis of human glioma.

Material and methods

Procurement of clinical tissues and
culturing and transfection of cell lines

The normal brain tissues adjacent to gliomas
and glioma tissues were taken from the patients
after their surgical excision at the Department of
Neurosurgery, The Fifth People’s Hospital of Shang-
hai, Fudan University, Shanghai, China. The pa-
tients were informed in advance and procurement
of clinical specimens was made only after written
consent had been signed by them. Standard sci-
entific ethical guidelines were followed strictly
for the experimental usage of these tissues. The
research ethics approval number for experimenta-
on human tissues was FUF/0881/2019. Liquid
nitrogen was used for the transportation of speci-
mens and their storage was done in ultra-low tem-
perature freezers. All the human glioma cell lines
(U87, MO59K, U118, Hs683 and LN18) along with
the normal astrocyte cell line were obtained from
the Cell Bank of the Chinese Academy of Science.
The culturing of all the cell lines was performed
using high glucose supplemented DMEM growth
medium (Thermo Fisher Scientific). A humidified
incubator was used for growing the cell lines at
37°C. Lipofectamine 2000 (Thermo Fisher Scientif-
ic) reagent was used to perform the transfection
of cell lines with specific transfection constructs.
The lncRNA-HULC silencing construct (si-HULC)
as well as the normal control silencing construct
were purchased from the RiboBio biotechnology
company.

RNA isolation and RT-PCR study

Using TRIzol reagent, the total RNA was isolat-
ed from the clinical tissues and cell lines. After its
treatment with DNase I and quantity estimation,
2.5 μg RNA was used to synthesize the cDNA with
the help of a high capacity cDNA synthesis kit
(Thermo Fisher Scientific). Then the expression
of lncRNA-HULC was analyzed using the One Step
SYBR Prime Script PLUS RT-RNA PCR Kit (TaKaRa
Biotechnology, Dalian, China) through the quan-
titative real time-PCR method. The relative ex-
pression levels were ascertained by 2–ddCt method
based calculations. The human β-actin gene was
used as an internal control in the expression anal-
ysis. The primers used are listed in Table I.

| Primer | Direction | Sequence |
|--------|-----------|----------|
| LncRNA | Forward   | 5’-TCATGATGGAGCCCTT-3’ |
|        | Reverse   | 5’-CCTCTTCCTGGCCTGAGATTG-3’ |
| β-actin| Forward   | 5’-CCTGGATAGCAACGTA-3’ |
|        | Reverse   | 5’-CACCTTCTACAATGAGCT-3’ |

Proliferation assay

The transfected glioma cells were added to 96-
well plates at the density of 2 × 10^5 cells/well. The

Table I. List of primers used in the study
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cells were cultured for either 0 h, 12 h, 24 h, 36 h, 48 h, 72 h or 96 h at 37°C. Cell proliferation rate was determined with the help of Cell Counting Kit-8 (CCK-8, Sigma-Aldrich). The absorbance was measured for each sample at 450 nm. The OD values were then used to draw the proliferation rate curves for their relative comparison.

**Analysis of cell apoptosis**

The transfected glioma cells were cultured for 48 h in 12-well plates at a density of $3 \times 10^5$ cells/well. Next, the cells were collected by centrifugation, washed with cold PBS, stained with 4',6-diamidino-2-phenylindole (DAPI) fluorescent staining solution and visualized under the fluorescent microscope for nuclear fluorescence to infer the level of apoptosis. The apoptosis levels were also analyzed through flow cytometry. Here the cells were stained with dual annexin V-FITC/PI staining mix prior to their flow cytometric investigation using the FITC Annexin V apoptosis detection kit (BD Bioscience).

**Cell migration and invasion assays**

For the analysis of migration of glioma cells transfected with specific transfection constructs, the cells were grown in 6-well plates at 37°C until the regular cell surface was obtained. Using the 200 µl pipette tip, the cell surface was perpendicularly scratched and photographed through a light microscope. The plate was incubated at 37°C for 24 h after which the scratch line was again observed under the microscope, photographed and compared with the initial scratch width. The invasion of transfected glioma cancer cells was determined using the transwell chamber plate fitted with Matrigel. After obtaining the homogeneous suspension by trypsinization, 250 µl of the suspension of transfected cells, with cellular density of $2.5 \times 10^6$ cells/ml, was added to the upper part of the transwell chamber. The lower part of the chamber was given 750 µl of growth medium only. The plate was incubated at 37°C for 48 h, and after the incubation, the cells invading the lower surface of the membrane were fixed with 70% ethanol, and then stained with Giemsa solution. The stained cells were visualized using an inverted microscope, photographed and the percentage of invasion was calculated using five random microscopic fields.

**Protein expression study**

The cell lysates were obtained by treating the transfected cells with RIPA lysis buffer. These extracts were separated on 10% SDS–PAGE gel which was blotted to PVDF membranes. Skimmed milk was used for blocking the membranes. Membranes were given the exposure of primary antibodies (Bax Cat#2774S, Bcl-2 Cat#42234S, PI3K Cat#4292, p-PI3K Cat#4228S, Akt Cat#9272, p-AKT Cat#4060 and β-actin Cat#4967, dilutions of 1 : 1000, Cell Signaling Technology, CST, USA) in dark at 4°C for 12 h, following which they were incubated with respective horseradish peroxidase conjugated secondary antibodies. With the help of enhanced chemiluminescence substrate, the protein bands were detected to infer their concentrations. Human β-actin protein was used to serve as the internal control in the protein expression studies.

**Statistical analysis**

The mean and standard deviation were calculated from the values obtained for a specific parameter and the final value was presented as mean ± standard deviation. The statistical analysis was performed using GraphPad prism 7.0 software. Student’s t test was performed and $p < 0.05$ was taken to represent a statistically significant level of difference between two values.

**Results**

**IncrRNA-HULC is highly expressed in glioma**

The qRT-PCR analysis was used to determine the expression of IncrRNA-HULC in the clinical tissues – normal and glioma. The results showed that glioma tissues exhibited significantly higher expression of IncrRNA-HULC (Figure 1 A). Similarly, the study of expression of IncrRNA-HULC from the glioma cell lines (U87, MO59K, U118, Hs683 and LN18) in comparison to normal astrocytes hinted towards the same inference, i.e., all the glioma cell lines showed significantly higher IncrRNA-HULC expression than the normal astrocytes (Figure 1 B). The cancer cell lines U87, MO59K, U118, Hs683 and LN18 respectively exhibited 3.7, 4.5, 4.3, 3.2 and 3.4 fold higher transcript levels of IncrRNA-HULC than normal astrocytes. Together, the results suggested the probable regulatory role of IncrRNA-HULC in human glioma.

**IncrRNA-HULC knockdown reduced proliferation of glioma cells through apoptotic induction**

To evaluate the effect of IncrRNA-HULC on proliferation of the glioma cells, knockdown of IncrRNA-HULC through transfection was carried out in two glioma cell lines, MO59K and U118. The relative expression of IncrRNA-HULC was only 0.2 and 0.18 in MO59K and U118 cell lines, respectively, in comparison to the negative control cells (transfected with si-NC). The silencing of IncrRNA-HULC was confirmed by the RT-PCR anal-
The cell counting kit-8 based estimation of proliferation assessment of cancer cells under lncRNA-HULC knockdown in comparison to the respective normal control cells showed that silencing of lncRNA-HULC actively decreased the proliferation of glioma cancer cells and the effects were more prominent when higher growth incubation periods were used (Figure 1 D). Now to look for the underlying basis of proliferation decline, a study of cell apoptosis was performed using DAPI staining. It was noted that the glioma cancer cells, MO59K and U118 showed clear signs of nuclear lesions under lncRNA-HULC knockdown indicating the induction of apoptosis (Figure 2 A). The induction of apoptosis in cancer cells under IncRNA-HULC gene silencing was also inferred from their flowmetric investigation. The percentage of apoptotic cells was significantly higher for the glioma cells under IncRNA-HULC knockdown in comparison to the normal control cells (Figure 2 B). The final support was gained from the blotting study of Bax and Bcl-2 apoptosis related proteins where Bax protein expression was seen to be enhancing under the knockdown of IncRNA-HULC while Bcl-2 was shown to be decreasing (Figure 2 C). The results thus clearly reveal that IncRNA-HULC gene

Figure 1. lncRNA-HULC is highly expressed in human glioma and its transcriptional knockdown markedly reduces the proliferation of glioma cells in vitro. A – Expression analysis of IncRNA-HULC in normal and glioma tissues. B – Expression analysis of IncRNA-HULC in normal astrocytes and glioma cancer cell lines (U87, MO59K, U118, Hs683 and LN18). C – Expression analysis of IncRNA-HULC in MO59K and U118 cancer cells transfected with si-NC or si-HULC. D – Cell counting kit-8 based assessment of proliferation of MO59K and U118 cancer cells transfected with si-NC or si-HULC. The experiments were performed in triplicate and expressed as mean ± SD (*p < 0.05)
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Silencing of lncRNA-HULC inhibited migration and invasion of glioma cancer cells

The effects of lncRNA-HULC knockdown were also studied on the glioma cancer cell motility. It was found that the silencing of lncRNA-HULC significantly reduced the migration of the glioma cancer cells M059K and U188 (Figure 3). The invasion of the glioma cancer cells was also observed to fall markedly when knockdown of lncRNA-HULC was carried out (Figure 4). Both the results depict that lncRNA-HULC might be controlling the metastasis of human glioma and its silencing might thus actively be able to check the disease spread.

Figure 2. LncRNA-HULC knockdown induces apoptosis in glioma cancer cells. A – DAPI staining for morphological assessment of nuclei of M059K and U118 cancer cells transfected with si-NC or si-HULC. B – Flow cytometric analysis for apoptosis of annexin V-FITC/PI dual stained M059K and U118 glioma cancer cells transfected with si-NC or si-HULC. C – Western blotting of Bax and Bcl-2 proteins from M059K and U118 cancer cells transfected with si-NC or si-HULC. The experiments were performed in triplicate.
The PI3K/AKT signaling pathway was blocked under lncRNA-HULC gene silencing

To try to determine the underlying mechanism through which lncRNA-HULC might be operating to regulate glioma cancer cell growth, western blotting of crucial components of the PI3K/AKT signaling pathway was performed. The results showed that when knockdown of lncRNA-HULC was made, the level of phosphorylated PI3K and AKT proteins significantly declined. However, little or no effect on the protein levels of non-phosphorylated forms of these proteins was seen (Figure 5). The finding thus suggests that lncRNA-HULC might be positively controlling the phosphorylation of PI3K and AKT proteins and its silencing might thus be used to partially block the PI3K/AKT pathway in glioma cells through inhibition of phosphorylation, which also highlights the therapeutic potential of lncRNA-HULC against human glioma growth and progression.

Discussion

During recent times the application of multiple anti-cancer procedures of surgical resection in combination with the current chemo- and radio-therapies has not only enhanced the survival rate of glioma but has also considerably reduced the suffering of glioma patients. However, satisfactory treatment results have not yet been achieved against this deadly disorder. As such, researchers are continuously in search of better therapeutic procedures, and in this regard, the exploration of various regulatory aspects controlling the growth and spread of human glioma is imperative. The current study was designed in the same direction and our results showed that aberrant expression of lncRNA-HULC might be one of the molecular errors governing the initiation and progression of glioma in humans, as has been proposed for this class of regulatory RNAs in human tumors [14–16]. Our study confirmed the over-expression of lncRNA-HULC in glioma, as was reported by previous research investigations. The knockdown of lncRNA-HULC reduced the proliferation of glioma cancer cells, which was confirmed to result from induction of apoptosis in glioma cells. The results are in line with the previous cancer cell growth reduction reports about lncRNA-HULC and support the proposition of therapeutic application of this
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RNA biomolecule against the human cancer [17, 18]. The treatment of human cancers including gliomas is hindered by some undesirable effects and majorly by the invasion of cancer cells to the surrounding tissues by the process of metastasis. Thus, it is crucial to evaluate measures which might restrict the cancer progression through metastasis. Here, in an interesting finding when the silencing of lncRNA-HULC was performed in the glioma cells, the cancer cells exhibited significantly lower motilities when investigated though in vitro assays. Such promising anti-cancer therapeutic results about the role of lncRNA-HULC in human cancers have also been noted by previous researchers [19–21]. Moreover, lncRNA-HULC through its interaction with miR-613 was shown to regulate the growth and metastasis of colon cancer cells in a similar fashion [22]. The sponging of miR-6754-5p by lncRNA-HULC was also reported to promote breast cancer development [23]. The cancer cells show higher growth potential because of the constitutive over-activation of signaling pathways governing the rate of division [24]. The PI3K/AKT signaling pathway is also among such pathways and thus workers have proposed the therapeutic targeting of this crucial pathway as a promising anti-cancer strategy [25]. In the present study, the results highlighted that the PI3K/AKT pathway

Figure 4. LncRNA-HULC knockdown reduces the in vitro invasion of glioma cancer cells. Transwell chamber assay for determination of invasion of M059K and U118 cancer cells transfected with si-NC or si-HULC. The experiments were performed in triplicate and expressed as mean ± SD (*p < 0.05)

Figure 5. PI3K/AKT signaling pathway is inhibited in glioma cancer cells under lncRNA-HULC gene silencing. Western blotting of phosphorylated PI3 and AKT (p-PI3 and p-AKT) proteins and their respective non-phosphorylated forms from the M059K and U118 cancer cells transfected with si-NC or si-HULC. The experiments were performed in triplicate
could be indirectly targeted and its signaling intensity might be lowered through transcriptional silencing of IncRNA-HULC. Regulation of the PI3K/AKT signaling pathway in glioma cells has already been confirmed by scientific researchers [26]. On the whole, the results of the current study support the regulatory role of IncRNA-HULC in human glioma together with the possibility of its usage as a vital prognostic and therapeutic molecule against the growth and progression of this aggressive disorder. Although the present study explored the therapeutic implications of LncRNA-HULC in glioma cells, the main limitation of the study is the lack of in vivo investigation. Therefore, the results of the present investigation require in vivo validation. Furthermore, identification of chemotherapeutic agents which can suppress the expression of LncRNA-HULC could open new avenues for the treatment of human glioma and remains an important area of investigation.

In conclusion, the findings of the present study are suggestive of significant up-regulation of IncRNA-HULC in human glioma. Interestingly, the results showed that experimental knockdown of IncRNA-HULC induced apoptosis in glioma cells and inhibited their growth and viability. LncRNA-HULC transcriptional silencing also remarkably hampered the migration and invasion of glioma cells. The regulatory effects of IncRNA-HULC were shown to be exerted through the PI3K/Akt signaling pathway. The findings point towards the therapeutic implications of IncRNA-HULC in glioma.

Conflict of interest

The authors declare no conflict of interest.

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