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ORIGINAL ARTICLE

Profiling and Bioinformatics Analyses of Hypoxia-Induced Differential Expression of Long Non-coding RNA in Glioblastoma Multiforme Cells

Xiaomin Cai[1] · Mengshu Qian[2] · Kui Zhang[3] · Yanzhen Li[1] · Bowen Chang[4] · Ming Chen[1]

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Abstract

Hypoxic microenvironments are intricately linked to malignant characteristics of glioblastoma multiforme (GBM). Long non-coding ribonucleic acids (lncRNAs) have been reported to be involved in the progression of GBM and closely associated with hypoxia. Nevertheless, the differential expression profiles as well as functional roles of lncRNAs in GBM cells under hypoxic conditions remain largely obscure. We explored the expression profiles of lncRNAs in hypoxic U87 cells as well as T98G cells using sequencing analysis. The effect of differentially expressed lncRNAs (DElncRNAs) was assessed through bioinformatic analysis. Furthermore, the expression of lncRNAs significantly dysregulated in both U87 and T98G cells was further validated using quantitative reverse-transcription polymerase chain reaction (qRT-PCR). Relevant cell functional experiments were also conducted. We used predicted RNA-binding proteins (RBPs) to construct an interaction network via the interaction prediction module. U87 and T98G cells showed dysregulation of 1115 and 597 lncRNAs, respectively. Gene Ontology (GO) analysis indicated that altered lncRNA expression was associated with nucleotide-excision repair and cell metabolism in GBM cells. Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis revealed the association between dysregulated lncRNAs and the Hippo signaling pathway under hypoxia. The dysregulation of six selected lncRNAs (ENST00000371192, uc003tnq.3, ENST00000262952, ENST00000609350, ENST00000610036, and NR_046262) was validated by qRT-PCR. Investigation of lncRNA-microRNA (miRNA)-mRNA networks centered on HIF-1α demonstrated cross-talk between the six validated lncRNAs and 16 related miRNAs. Functional experiments showed the significant inhibition of GBM cell proliferation, invasion, and migration by the knockdown of uc003tnq.3 in vitro. Additionally, uc003tnq.3 was used to construct a comprehensive RBP-transcription factor (TF)-miRNA

Xiaomin Cai, Mengshu Qian, Kui Zhang and Yanzhen Li have contributed equally to this work.

Extended author information available on the last page of the article

interaction network. The expression of LncRNAs was dysregulated in GBM cells under hypoxic conditions. The identified six lncRNAs might exert important effect on the development of GBM under hypoxic microenvironment.

Keywords Sequence analysis · LncRNA · Expression profile · LncRNA-miRNA-mRNA network

Abbreviations

GBM	Glioblastoma multiforme
lncRNA	Long non-coding ribonucleic acid
HIF-1α	Hypoxia-induced factor-1α
qRT-PCR	Quantitative reverse-transcription polymerase chain reaction
GO	Gene Ontology
KEGG	Kyoto Encyclopedia of Genes and Genomes
ncRNA	Non-coding ribonucleic acid
ceRNA	Competing endogenous RNA
miRNA	MicroRNA
DElncRNA	Differentially expressed lncRNA
siRNA	Small interfering RNA
CCK-8 assay	Cell counting kit-8 solution assay
OD	Optical density
one-way ANOVA	One-way analysis of variance
FC	Fold change
BP	Biological process
CC	Cellular component
MF	Molecular function
UHRF1	Ubiquitin-like with PHD and RING finger domains 1
UPAT	UHRF1-protein associated transcript
HIE	Hypoxic ischemic encephalopathy
IGFBP3	Insulin-like growth factor binding protein 3
RBP	RNA-binding protein
TF	Transcription factor

Introduction

Glioblastoma multiforme (GBM) is a common primary central nervous system tumor (Alifieris and Trafalis 2015). The current standard treatment involves complete surgical resection followed by postoperative radiation and chemotherapy; however, the prognosis for GBM patients remains poor (Rhun et al. 2019; Touat et al. 2017). Hypoxia can be caused by both pathological and physiological conditions (Amjadi et al. 2023). The hypoxic microenvironment is closely linked to tumorigenesis, angiogenesis, invasion, as well as resistance to radiotherapy, leading to high mortality rates among GBM patients. Previous research suggests that hypoxia may

be an important therapeutic target and an influencing factor in GBM (Domenech et al. 2021; Colwell et al. 2017).

Non-coding ribonucleic acids (ncRNAs), particularly microRNAs and long non-coding RNAs (lncRNAs), play crucial roles in various physiological and cellular processes (Adams et al. 2017). LncRNAs exert their functions through diverse mechanisms of action, such as regulating transcription, epigenetic modifications, protein/RNA stability, translation, as well as posttranslational modifications by interacting with DNA (Postepska-Igielska et al. 2015), RNAs (Zealy et al. 2018), and/or proteins (Yamazaki et al. 2018). In addition, lncRNAs can serve as oncogenes / tumor suppressors within GBM (Peng et al. 2018). Importantly, numerous studies have demonstrated that HIF-1α has the ability to regulate lncRNAs in different tumor types, including gliomas (Bischoff et al. 2017; Chang et al. 2016). Consequently, lncRNAs hold great promise as potential therapeutic targets for GBM under hypoxic conditions.

In the present study, we were aimed to elucidate the functions and expression patterns of lncRNAs in hypoxic GBM. To investigate hypoxia-induced (DElncRNAs) in GBM, we conducted sequencing analysis. Subsequently, bioinformatics analysis was conducted for predicting the roles along with the associated pathways of DElncRNAs. Dysregulated lncRNAs in T98G and U87 cells were identified and confirmed using quantitative reverse-transcription polymerase chain reaction (qRT-PCR), and relevant functional experiments were conducted. Additionally, we established interaction networks between the selected lncRNA-associated RNA-binding proteins (RBPs), transcription factors (TFs), and miRNAs. Overall, this study was aimed to identify DElncRNAs in hypoxic GBM cells and explore their potential functions and regulatory networks.

Materials and Methods

Cell Culture

The U87 and T98G cell lines were obtained from the Cell Bank of the Chinese Academy of Sciences. The culture media for U87 and T98G cells comprised Dulbecco's Modified Eagle Medium (Gibco), 10% fetal bovine serum (Gibco), and 1% penicillin–streptomycin (Gibco). For the normoxic assay, cells were cultured at 37 °C with 5% CO_2, while for the hypoxic assay, cells were cultured at 37 °C with 5% CO_2 and 1% O_2. Three pairs of U87 cells under hypoxic (U87T) and normoxic (U87C) conditions, as well as three pairs of T98G cells under hypoxic (T98GT) and normoxic (T98GC) conditions, were prepared for sequencing analysis and subsequent experiments.

qRT-PCR

The qRT-PCR was performed as described previously (Chen et al. 2022). Briefly, total RNA was extracted using TRIzol reagent, reverse transcribed to complementary

DNA, and then subjected to qRT-PCR using an Applied Biosystems 7500 Fast Real-Time PCR System. All samples underwent three independent assays. The $2^{-\Delta\Delta CT}$ method was used to measure lncRNA levels, with Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) serving as a reference for normalization. The primer sequences for selected lncRNAs are provided in Supplementary Table 1.

LncRNA Sequencing Analysis

We obtained lncRNAs from various sources, including specific lncRNA databases, diverse genome annotation programs, and lncRNA-associated studies. These sources included RefSeq (https://www.ncbi.nlm.nih.gov/refseq/), UCSC_KnownGene (http://genome.ucsc.edu/cgibin/hgGateway), Gencode (https://www.gencodegenes.org/), Ensembl (http://asia.ensembl.org/index.html), lncRNAdb (https://lncipedia.org/), lncRNA disease (http://www.cuilab.cn/lncrnadisease), GenBank (https://www.uniprot.org/database/DB0028), RNAdb (https://rnacentral.org/), and NRED (http://jsm.research.imb.uq.edu.au/nred/). Cloud Seq Pte Ltd. was responsible for preparing RNA libraries and conducting high-throughput sequencing. RNA libraries were prepared using the TruSeq stranded total RNA library prep kit (Illumina), followed by RNA extraction with TRIzol reagent (Thermo Fisher Scientific) as per specific protocols. Quantitative library analysis and quality control were performed using a BioAnalyzer 2100 system (Agilent Technologies). The libraries (10 pM) were denatured to single-stranded DNA and captured using Illumina Flowcells (Illumina). Cluster amplification was performed in situ, followed by 150-cycle sequencing in two terminal modes (PE modes) with the Illumina Novaseq 6000 sequencing equipment (Illumina) according to specific instructions.

Analysis of lncRNA Profiles

Paired-end reads were obtained using the Illumina Novaseq 6000 sequencing equipment, and Q30 was used for quality control (Liu et al. 2020). Low-quality reads were removed using Cutadapt software (v1.9.3) to ensure only high-quality reads remained (Kechin et al. 2017). Hisat2 software (v2.0.4) (http://ccb.jhu.edu/software/hisat2/index.shtml) was employed to align the high-quality reads to the human reference genome. Cuffdiff software (v2.2.1) was used to determine the value of fragments per kilobase of exon per million fragments mapped for each individual lncRNA according to the description in the Gene Transfer Format annotation file. Moreover, the statistical factors of DElncRNAs were screened by differences in thresholds ($p < 0.05$) and fold change (FC) (2.0) between the control and experimental groups.

The identified DElncRNAs were later subjected to Gene Ontology (GO) (http://www.geneontology.org) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (https://www.genome.jp/kegg/) analyses to predict their possible functions (Kanehisa et al. 2019). To better understand the functional mechanisms associated with genes linked to DElncRNAs, the genes from the two distinct cell lines were separated into upregulated and downregulated groups for GO or KEGG analysis. GO functions were

classified into three subcategories: Biological Process (BP), Cellular Component (CC), and Molecular Function (MF). KEGG pathway analysis was conducted to investigate the biological pathways in which DElncRNAs might be involved. The top 10 GO terms or KEGG pathways with the highest Enrichment Score were selected.

Construction of Predicted HIF-1α Centered LncRNA-miRNA-mRNA Interaction Networks

The identified DElncRNAs were used to predict potential miRNA response elements and the binding sites of miRNAs using CloudSeq's in-house software based on miRanda and TargetScan (CloudSeq Inc., China). The network between lncRNAs and miRNAs was constructed using Cytoscape based on the binding sites of the DElncRNAs and miRNAs. Different nodes represented the DElncRNAs, HIF-1α, and miRNAs, each distinguished by color. Solid lines indicated the potential binding between two nodes.

Construction of RBP-TF-miRNA Interaction Networks

The catRAPID omics (http://service.tartaglialab.com/page/catrapid_omics_group) was used to predict potential RBPs related to uc003tnq.3. The RBP-TF-miRNA networks were constructed using the online tool NetworkAnalyst (https://www.networkanalyst.ca/) based on the ENCODE ChIP-seq data (https://www.encodeproject.org/) and miRTarBase v8.0 (https://mirtarbase.cuhk.edu.cn/php/download.php).

Silencing of uc003tnq.3

Three small interfering RNAs (siRNAs) and a negative siRNA control (si-NC) were synthesized (GenePharma, China). The targeting sequences for si-uc003tnq.3#1, si-uc003tnq.3#2, si-uc003tnq.3#3, and si-NC were 5'-CAAGAAAGGGCATGCTATT-3', 5'-CCACTGACTTTGTGACTTT-3', 5'-AGGTTAATGTGGAGCTCTT-3', and 5'-UUCUCCGAACGUGUCACTT-3', respectively. The siRNAs targeting the junction site of uc003tnq.3 and si-NC were transfected into U87 and T98G cells using Lipofectamine 2000 as per the protocol. After 48 h, the interference efficiency was evaluated by qRT-PCR. The siRNA with the highest silencing efficiency was selected for subsequent experiments.

Cell Viability Assay

Cell viability and proliferation were assessed using the Cell Counting Kit-8 solution (CCK-8) assay (Dojindo, Japan) following the previously described method (Cai et al. 2022).

Wound Healing Assay

The glioma cells that had been appropriately treated were seeded in 6-well plates at a density of 3×10^5 cells/well and cultured at 37 °C until they reached a confluence of at least 90%. A sterile 200 µL pipette tip was used to create a scratch on the cell monolayer, and the wound area was photographed at 0 and 24 h under an inverted light microscope (Olympus, Japan). The mean number of migrating cells was then calculated.

Transwell Invasion Assay

The transwell invasion assay was conducted using 24-well BD Matrigel Invasion Chambers (BD Biosciences) according to a previously described method (Cai et al. 2020). Briefly, suitably treated 1×10^5 cells were seeded in the upper chamber with serum-free medium. The lower chamber wells were supplemented with medium containing 10% fetal bovine serum (FBS) as a chemo-attractant. Following incubation for 24 h, non-invading cells were removed, and the invading cells on the bottom were fixed with methanol and stained with 0.1% crystal violet for 15 min. The number of invading cells was counted from 5 randomly chosen fields per chamber using an inverted light microscope (Olympus, Japan).

Statistical Analysis

Data analysis was performed using GraphPad Prism 5.0 software (GraphPad Software, USA). Normally distributed variables were compared between two groups using Student's unpaired t test, while abnormally distributed variables were compared using the Mann–Whitney *U* test. One-way analysis of variance (ANOVA) was used to compare multiple groups, and pairwise comparisons across several groups were performed using ANOVA plus Tukey's test. Pearson's correlation coefficient was used to determine associations among variables. Each assay was performed in triplicate. *P* value less than 0.05 was considered statistically significant.

Results

RNA-seq Profiling of lncRNAs in GBM Cells Under Hypoxia and Normoxia

DElncRNAs in U87 and T98G cells under hypoxia and normoxia were observed using scatter plots. Additionally, volcano plots were created to show the DElncRNAs selected based on the thresholds of FC (≥ 2.0) and p value (< 0.05) in U87 and T98G cells under both hypoxic and normoxic conditions. Among the 1115 dysregulated lncRNAs identified in U87 cells under hypoxia, 580 lncRNAs were upregulated and 535 lncRNAs were downregulated (the top 10 upregulated and downregulated lncRNAs sorted by Log_2FC are shown in Supplementary Table 2). In T98G cells, among the 597 dysregulated lncRNAs identified under hypoxia, 139 lncRNAs

were upregulated and 458 lncRNAs were downregulated (the top 5 upregulated lncRNAs and top 10 downregulated lncRNAs sorted by Log_2FC are shown in Supplementary Table 3). A cluster heatmap clearly displayed the DElncRNAs in these two GBM cell lines between hypoxic and normoxic conditions (Fig. 1). The complete list of all DElncRNAs is provided in Supplementary Appendix 1, along with important information such as transcript ID, fold change, and false discovery rate.

Prediction of lncRNA Functions within GBM Cells Under Hypoxia

In U87 cells, Fig. 2 and Supplementary Table 4 present the top ten enriched biological processes (BP), cellular components (CC), and molecular function (MF) terms, as determined by their respective enrichment scores. The top three upregulated lncRNA-associated BPs were specifically associated with nucleotide-excision repair. Concerning CC terms, they were predominantly linked to the H4 histone acetyltransferase complex, nucleotide-excision repair complex, and cell junctions. Notably, the top two MF terms were cation antiporter activity and metal ion transmembrane transporter activity. Furthermore, KEGG analysis revealed that the top three pathways associated with the upregulated lncRNAs included nucleotide-excision repair, protein digestion and absorption, and pancreatic secretion (Fig. 3 and Supplementary Table 5).

In T98G cells, the top three upregulated lncRNA-associated BPs were characterized by their response to herbicide, positive regulation of the mitogen-activated protein kinase (MAPK) cascade, and MAPK cascade itself. As for CC terms, they predominantly pertained to the cholinergic synapse, growth cone, and sites of polarized growth. Regarding MF terms, they were primarily associated with enzyme activator activity, alcohol dehydrogenase [NAD (P) +] activity, and

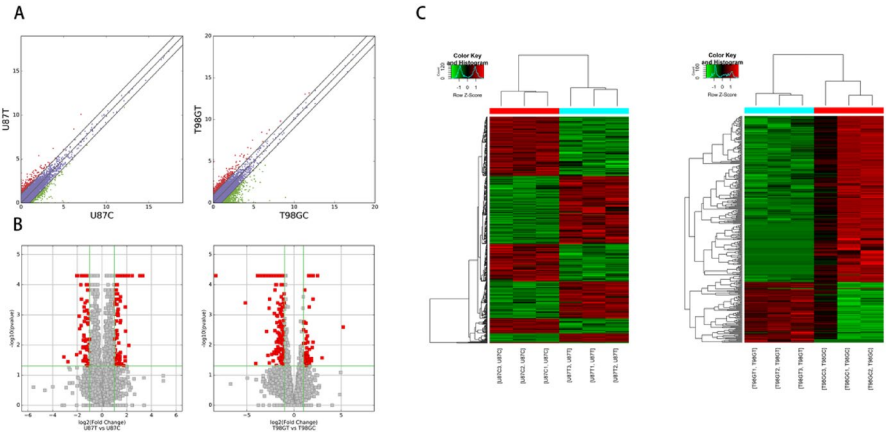

Fig. 1 Different expression profiles of lncRNAs in GBM cell lines between hypoxic and normoxic conditions. **A** Scatter plots showed the dysregulated lncRNAs in U87 and T98G cells under hypoxia. **B** Volcano plots showed the DElncRNAs in U87 or T98G cells under hypoxia. **C** Hierarchical clustering expressed by analyzing DElncRNAs in U87 and T98G cells between under hypoxia and normoxia, with green and red strips representing downregulation and upregulation, separately

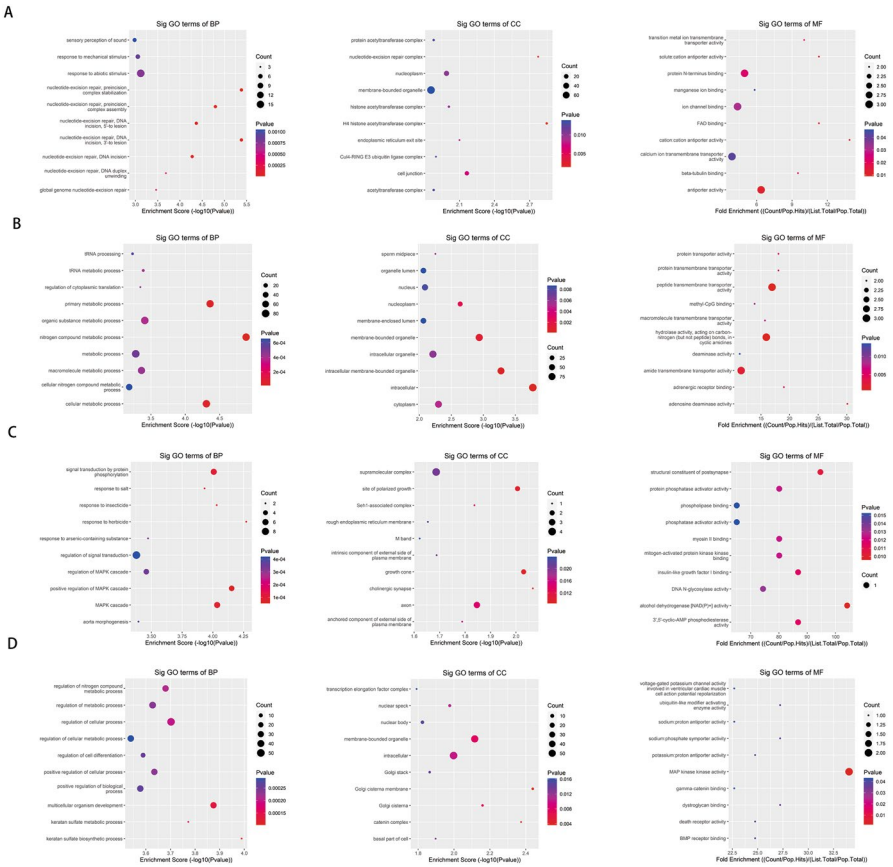

Fig. 2 GO analysis on DElncRNAs host genes in GBM cell lines under hypoxia. **A–D** GO analysis results of upregulated **A**, **C** or downregulated **B**, **D** lncRNAs host genes in U87 **A**, **B** or T98G cells **C**, **D** under hypoxic conditions

structural constituents at the post-synapse (Fig. 2 and Supplementary Table 6). Similarly, KEGG analysis suggested that the top three pathways relevant to the upregulated lncRNAs were the Hippo signaling pathway, tight junction pathway, and tuberculosis pathway (Fig. 3 and Supplementary Table 7).

The downregulated lncRNAs were analyzed using the same methodology. In U87 cells, the top three downregulated lncRNA-associated BPs were nitrogen compound metabolic process, cellular metabolic process, and primary metabolic process. The top three CC terms were intracellular, membrane-bounded organelle, and intracellular membrane-bounded organelle. Furthermore, the top three MF terms were peptide transmembrane transporter activity, nucleosome binding, and hydrolase activity (Fig. 2 and Supplementary Table 4). KEGG analysis revealed that the top three pathways associated with the downregulated lncRNAs

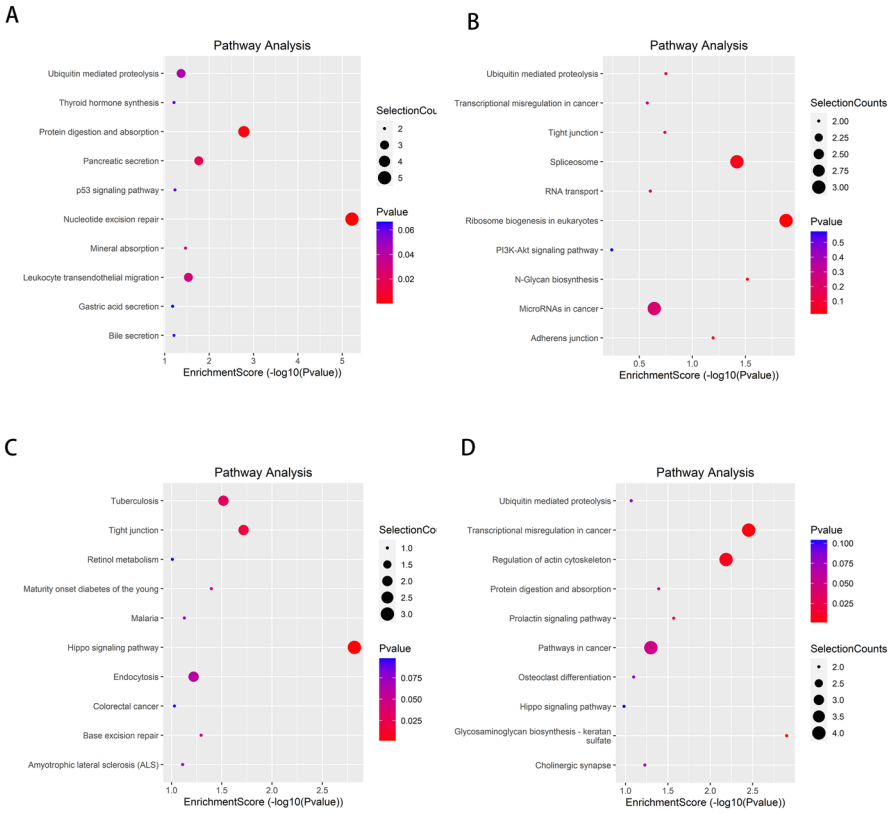

Fig. 3 KEGG analysis of DElncRNAs host genes in GBM cell lines under hypoxia. **A**–**D** Top 10 pathways shown by KEGG analysis in upregulated **A**, **C** or downregulated **B**, **D** lncRNAs host genes in U87 **A**, **B** or T98G cells **C**, **D** under hypoxic conditions

were ribosome biogenesis in eukaryotes, N-glycan biosynthesis, and spliceosome (Fig. 3 and Supplementary Table 5).

In T98G cells, the top three downregulated lncRNA-associated BPs were keratan sulfate biosynthetic process, multicellular organism development, and keratan sulfate metabolic process. Regarding CC terms, they were primarily related to the Golgi cisterna membrane, catenin complex, and Golgi cisterna. The top three MF terms included MAPK kinase activity, Rac GTPase binding, and Rho GTPase binding (Fig. 2 and Supplementary Table 6). KEGG analysis indicated that the top three pathways associated with the downregulated lncRNAs were glycosaminoglycan biosynthesis-keratan sulfate, regulation of actin cytoskeleton, and transcriptional misregulation in cancer (Fig. 3 and Supplementary Table 7).

Validation of the DElncRNAs in GBM Cell Lines Under Hypoxia

Among all the identified lncRNAs, we selected the dysregulated lncRNAs that were consistently observed in both U87 and T98G cells under hypoxia. LncRNAs without corresponding mRNA were excluded. Our results indicated that two lncRNAs were upregulated, and four lncRNAs were downregulated in both U87 and T98G cells under hypoxia (Fig. 4A, B). Subsequently, these six lncRNAs were subjected to qRT-PCR validation using divergent primers. The expression levels of the six selected lncRNAs were detected via qRT-PCR. As depicted in Fig. 4C, the expressions of ENST00000371192 and uc003tnq.3 were significantly higher in both U87 and T98G cells under hypoxia compared to normoxia. Additionally, the expression levels of ENST00000262952, ENST00000609350, ENST00000610036, and NR_046262 were lower in both U87 and T98G cells under hypoxia in contrast with normoxia with statistical significance (Fig. 4D).

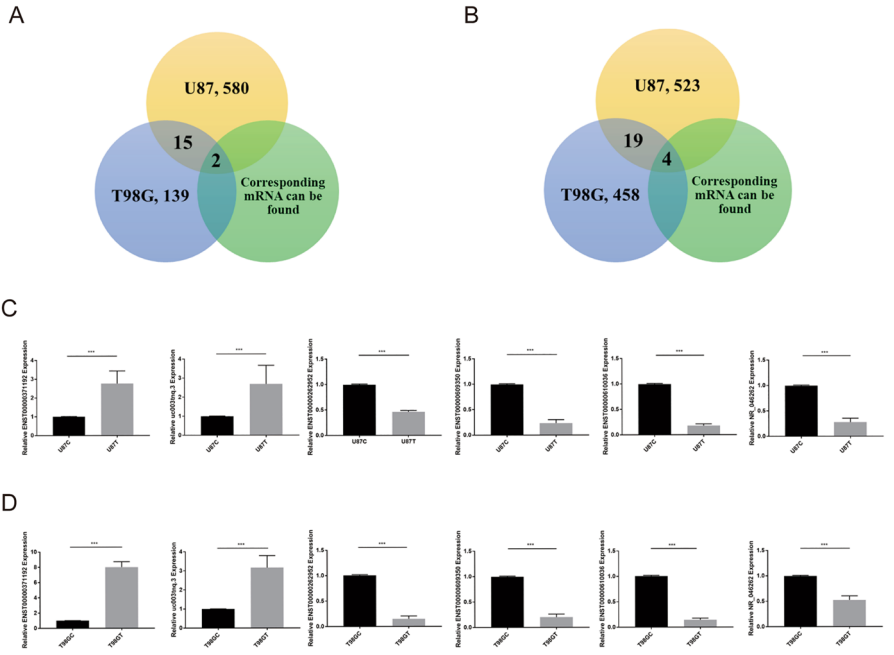

Fig. 4 Verification of the DElncRNAs in GBM cells under hypoxia. **A**, **B** The distribution of upregulated **A** or downregulated **B** lncRNAs from different catalogs in U87, T98G, and corresponding mRNA was shown. **C**, **D** Six of the selected LncRNAs were examined through qRT-PCR. The relative expression levels of lncRNAs in U87 **C** and T98G cells **D** were shown. ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.)

Establishment of lncRNA-miRNA-mRNA Interaction Networks

Considering this study paid attention to hypoxic conditions, we centered our analysis around HIF-1α. Meanwhile, we adopted six lncRNAs validated by qRT-PCR to establish a lncRNA-miRNA-mRNA interaction network for prediction (Fig. 5). Different nodes with distinct colors represented the DElncRNAs, HIF-1α, and miRNAs, respectively.

Silencing of uc003tnq.3 Inhibited GBM cell's Proliferation, Invasion, and Migration Under Hypoxia

To investigate the potential impact of the loss of uc003tnq.3 on the progression of GBM under hypoxic conditions, we initially infected GBM cells with three independent siRNAs or a control siRNA. The altered expression of uc003tnq.3 in GBM cells was subsequently confirmed using qRT-PCR. The si-uc003tnq.3#2 was chosen for further investigation considering its higher efficiency in knockdown (Fig. 6A). Subsequently, we assessed the impact of uc003tnq.3 on cellular proliferation in U87 cells as well as T98G cells under hypoxia using the CCK-8 assay. The growth rates of uc003tnq.3-depleted U87 and T98G cells were lower in contrast with those of the control cells with statistical significance (Fig. 6B). Additionally, we examined the impact of uc003tnq.3 knockdown on the invasion and migration of glioma cells under hypoxia by conducting transwell and wound healing assays, respectively. As depicted in Fig. 6C, the knockdown of uc003tnq.3

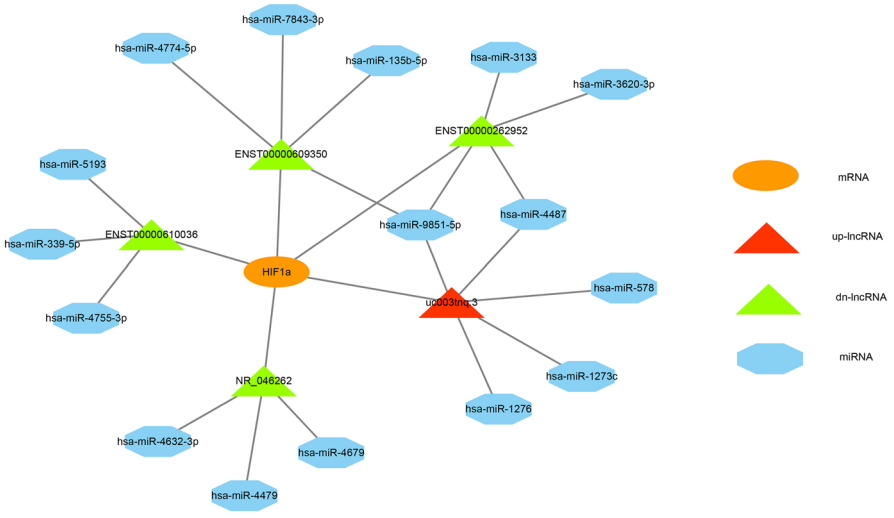

Fig. 5 Construction of lncRNA-miRNA-mRNA interaction networks. The predicted HIF-1α centered lncRNA-miRNA-mRNA interaction networks were established. The red triangles represented upregulated lncRNAs. The green triangles represented downregulated lncRNAs. The blue octagons represented miRNAs. ($^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, $^{****}p<0.0001$)

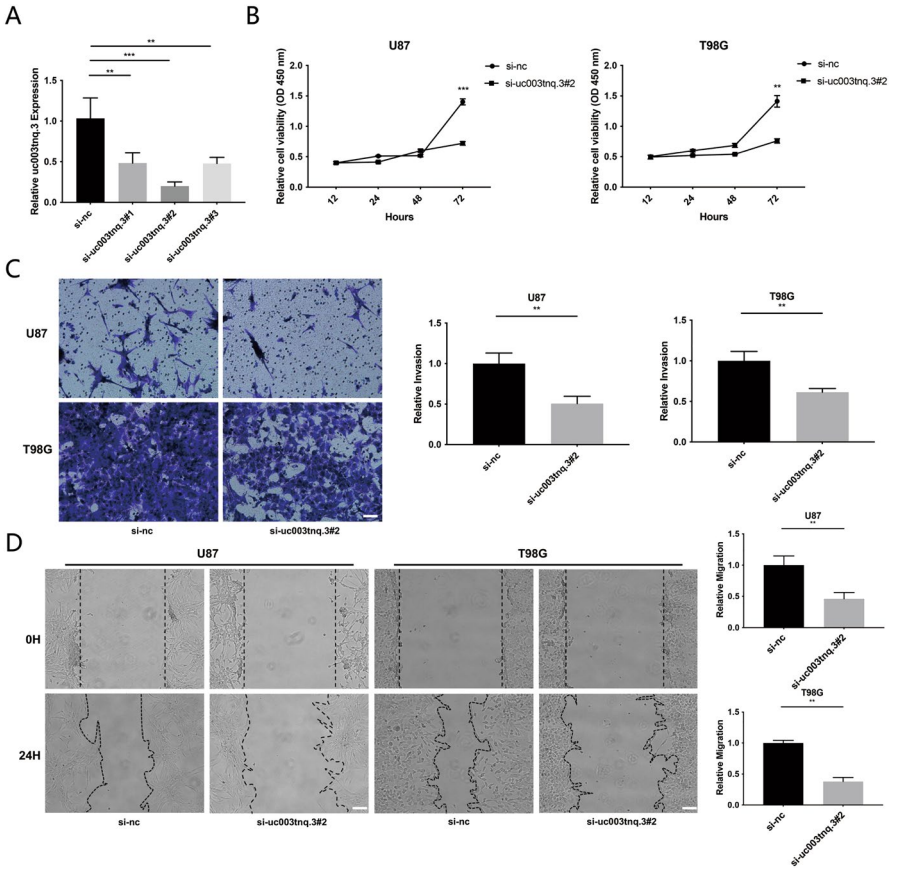

Fig. 6 Silencing of uc003tnq.3 blocks GBM cell's proliferation, invasion, and migration under hypoxia. **A** The knockdown efficiency of indicated siRNAs was measured by using qRT-PCR after 48 h. **B** The proliferation ability of U87 and T98G cells under hypoxia transfected with si-uc003tnq.3#2 or control siRNA was determined by CCK8 assay ($n=3$). **C**, **D** Cell invasion and migration ability of U87 and T98G cells under hypoxia transfected with si-uc003tnq.3#2 or control siRNA were determined by transwell assay (**C**) and wound healing assay (**D**), respectively. ($^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, $^{****}p<0.0001$)

led to a marked reduction in the number of invading cells under hypoxic conditions compared to the controls. Similarly, the wound healing assay demonstrated a similar trend (Fig. 6D). Overall, these findings indicate that the knockdown of uc003tnq.3 effectively inhibits cell proliferation, invasion, and migration of glioma cells under hypoxic conditions.

RBP-TF-miRNA Interactions and Networks

RBPs are a diverse group of proteins that interact with various types of RNAs, including microRNAs (miRNAs) and lncRNAs, through specific structural motifs

and domains (Yao et al. 2022). These interactions play critical roles in RNA processing, modifications, stability, and function (Jonas et al. 2020). In recent years, numerous studies have revealed dysregulated RBPs and their interacting lncRNAs in various human cancers, highlighting their potential contributions to tumor development and progression[20}. For instance, the RBP SRSF1 is involved in maintaining the stability of the nuclear-enriched lncRNA NEAT1, thereby regulating the cell cycle in glioma (Zhou et al. 2019). Therefore, our next objective was to identify the RBPs associated with uc003tnq.3. The complete nucleotide sequence of uc003tnq.3 can be found in Supplementary Appendix 2. Through the utilization of the catRAPID omics analysis tool (http://service.tartaglialab.com/page/catrapid_omics_group), we identified over 10,000 RBPs that potentially interact with uc003tnq.3. The top 15 RBPs can be found in Supplementary Appendix 3. Among the top 15 RBPs associated with uc003tnq.3, we selected four RBPs (TIA1, SRSF3, A1CF, and FUS) for enrichment analysis and constructed a comprehensive network that also included TFs and miRNAs. This RBP-TF-miRNA network included 129 nodes as well as 129 edges (Fig. 7A). Enrichment analysis based on the hub RBPs and predicted TFs and miRNAs revealed associations with transcription regulator complexes, DNA-binding transcription activator or repressor activities, and transcriptional misregulation in cancer (Fig. 7B).

Discussion

Gliomas are prevalent primary brain tumors, with GBM being the most aggressive pathological type among them (Linhares et al. 2020). Despite the utilization of standard therapeutic approaches, the prognosis for GBM remains bleak, with a median survival time of 14–16 months. Extensive research has demonstrated that the hypoxic microenvironment plays a critical role in the malignant progression, drug resistance, and prognosis of GBM. However, there is currently a lack of studies investigating the expression profile of lncRNAs in GBM under hypoxic conditions. In this study, we conducted sequence analysis to identify a multitude of DElncRNAs in two GBM cell lines subjected to hypoxia. Subsequently, we validated the expression of six selected lncRNAs at the mRNA level. Furthermore, we carried out the functional experiments, aiming to explore the role of one specific lncRNA in GBM.

There is mounting evidence suggesting that lncRNAs play a significant role in regulating tumor progression under hypoxic conditions. For instance, the hypoxia-induced lncRNA GHET1 has been shown to lead to excessive activation of the Hippo/YAP signaling pathway, thus promoting the progression of triple-negative breast cancer (Wang and Liu 2021).Additionally, the hypoxia-inducible lncRNA-AC020978 has been found to promote the proliferation as well as the glycolytic metabolism of non-small cell lung cancer (NSCLC) through the regulation of the PKM2/HIF-1α axis (Hua et al. 2020). Moreover, the expression of lncRNA H19 has been observed to be stimulated under hypoxia, exerting malignant effects in glioblastomas (Wu et al. 2017). However, limited research has focused on alternations in the expression profile of lncRNAs among glioblastoma cell lines under hypoxic conditions. In our study, we selected the U87 and T98G cell lines as subjects and

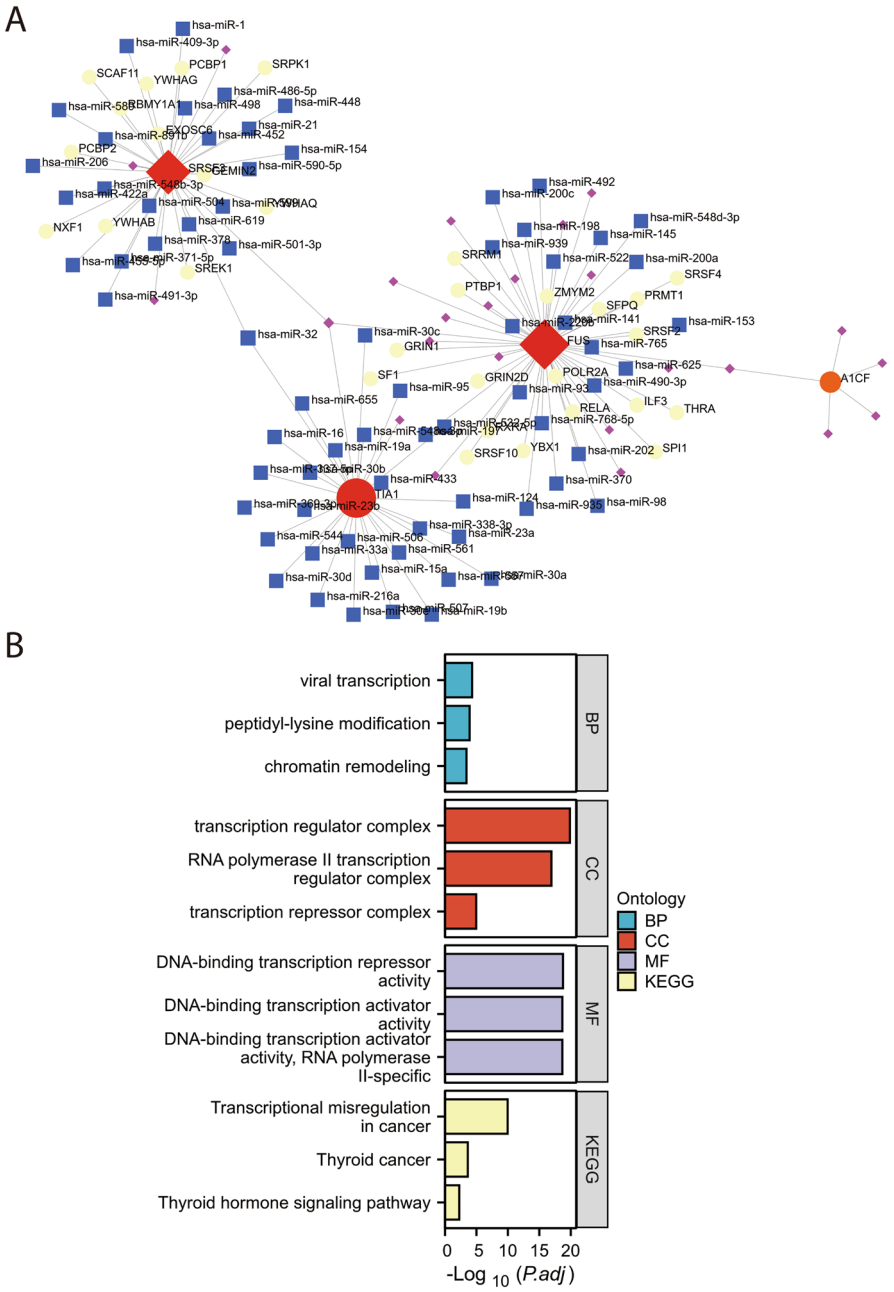

Fig. 7 RBP-TF-miRNA interactions and networks. **A** The 4 RBPs were represented by red spot, red rhombuses, and orange spot, respectively. TFs and miRNAs were represented by blue squares. **B** GO and KEGG analysis for the RBP-TF-miRNA interaction network were shown in the form of a histogram

aimed to examine differentially expressed lncRNAs between normoxia and hypoxia culture conditions. These two cell lines are commonly employed as targets for research on chemical drugs in GBM treatment (Santangelo et al. 2020; Bilal et al. 2021). Our analysis identified a total of 1115 dysregulated lncRNAs under hypoxia in U87 cells and 597 dysregulated lncRNAs in T98G cells. These findings serve as a preliminary foundation for subsequent research on GBM under hypoxic conditions. A similar study by Jennifer Koehler et al. compared miRNA expression in hypoxic and normoxic conditions in three canine glioma cell lines (J3T, SDT3G, and G06A cell lines), identifying 21 differentially regulated miRNAs across all three cell lines (Koehler et al. 2020). Furthermore, Yue Qin et al. developed a hypoxia-associated lncRNA prognostic model to predict the outcomes of GBM patients (Qin et al. 2023). However, their conclusions were derived from public databases, including The Cancer Genome Atlas database and the Molecular Signature Database. This differs from the methodology employed in our study, suggesting a certain degree of novelty and potential for inspiring subsequent related research.

GO and KEGG analyses were conducted to validate the top ten GO terms and pathways associated with the DElncRNAs identified in GBM under hypoxic conditions. The results of BP and MF analyses revealed that the DElncRNAs were predominantly involved in nucleotide-excision repair and cell metabolism, highlighting their significant roles in GBM under hypoxia. A previous study by Yunfei Liao et al. demonstrated that protein arginine methyltransferase 3 drives the malignant progression of GBM by enhancing glycolytic metabolism, underscoring the importance of cell metabolism in this context (Liao et al. 2022). Interestingly, KEGG analysis also indicated that metabolism is one of the three pathways closely associated with the DElncRNAs in GBM cells under hypoxia. Regarding CC analysis, notable differences were observed between U87 and T98G cells. The top three CC terms in U87 were related to the H4 histone acetyltransferase complex, nucleotide-excision repair complex, and cell junctions. In contrast, the top three CC terms in T98G were the cholinergic synapse, growth cone, and sites of polarized growth. These dissimilarities are possibly related with the distinct characteristics of these two cell lines, such as the photodynamic treatment resistance exhibited by T98G cells (Bilal et al. 2021).

Functional experiments were conducted to validate the reliability of the data for lncRNA sequencing analysis. We selected and validated six dysregulated lncRNAs that were found in both U87 and T98G cells under hypoxia. The differential expression of these lncRNAs in GBM cell lines suggests their potential regulatory roles as either tumor-promoting or tumor-inhibiting lncRNAs. Currently, studies have proved that lncRNAs can serve as new prognostic biomarkers as well as therapeutic targets (Fattahi et al. 2020; Amer et al. 2022). In our study, we observed hypoxia-induced downregulation of ENST00000262952, which targeted the ubiquitin-like with PHD and RING finger domains 1 (UHRF1) gene, in GBM cells. It suggests that ENST00000262952 might play a role in the malignant phenotype of GBM under hypoxia. Additionally, it has been reported that the lncRNA UHRF1-protein-associated transcript (UPAT) is overexpressed in various types of cancers and can enhance cell growth by up-regulating UHRF1 levels in NSCLC (Wang et al. 2018). Furthermore, we found significant downregulation of NR_046262, which targets the NIPBL gene, in GBM cells under hypoxic conditions. Studies have

indicated that high NIPBL levels are associated with poor prognosis in NSCLC (Xu et al. 2015). Another study suggested that the transcription of both NIPBL and the 5.3 kb lncRNA NIPBL-AS1 is controlled by a bidirectional promoter, highlighting the effect of lncRNAs on NIPBL expression (Zuin et al. 2017). All these results supported the effect of lncRNAs on NIPBL expression. In contrast, we observed significant upregulation of uc003tnq.3, which targets the insulin-like growth factor binding protein 3 (IGFBP3) gene, in both U87 and T98G cells under hypoxia. Previous studies on neonatal hypoxic ischemic encephalopathy (HIE) have identified a novel lncRNA that specifically targets the IGFBP3 gene, and it has been found to be enriched in cell growth and cell apoptosis processes (Xiong et al. 2020). Silencing IGFBP3 has been demonstrated to inhibit the survival and growth of neurons in HIE rats, promote cell apoptosis, and consequently lead to motor and cognitive function defects (Xiong et al. 2020). Since research on IGFBP3 has already been reported, we decided to conduct an in-depth study on uc003tnq.3. Our findings showed that downregulation of uc003tnq.3 obviously suppressed the proliferation, invasion, as well as migration of GBM cells under hypoxia, which aligns with previously reported results (37). However, further investigation is needed to determine the participation of ENST00000262952, NR_046262, ENST00000371192, ENST00000609350, and ENST00000610036 in GBM tumorigenesis and their underlying mechanisms under hypoxic conditions.

This was a preliminary study, with certain limitations necessary to be acknowledged. Firstly, distinct GBM cell lines possibly exhibit varied responses to hypoxia. In the present study, we focused on validating the DElncRNAs identified in both U87 and T98G cells. However, other GBM cell lines, like LN229 and U373, have not been included in our investigation, and their specific reactions to hypoxic conditions have remained unknown. Secondly, this study's sample size was relatively small. Therefore, future research should expand the sample size and incorporate additional cell lines to allow for a more comprehensive analysis of DElncRNAs. Furthermore, it is essential to verify these findings at the fundamental research level.

Conclusions

In conclusion, our study demonstrated DElncRNAs in GBM cell lines under hypoxic conditions. These findings suggest that lncRNAs, particularly the six validated lncRNAs in this study, may exert pivotal effects on the development and progression of GBM under hypoxia. Consequently, they hold potential as prognostic markers as well as adjunct therapeutic targets for GBM. Nevertheless, further research is needed to gain a deeper understanding of the molecular mechanisms underlying these findings.

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Author contributions MC and BWC designed and supervised the project. XMC, MSQ, KZ, and YZL conducted the experiments and performed data analysis. BWC and XMC wrote and revised the manuscript.

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Availability of data and materials The raw data supporting the conclusion of this article have been uploaded to Supplementary Appendix 4.

Declarations

Competing interests The authors declare no competing interests.

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Authors and Affiliations

Xiaomin Cai[1] · Mengshu Qian[2] · Kui Zhang[3] · Yanzhen Li[1] · Bowen Chang[4] · Ming Chen[1]

✉ Bowen Chang
changbowen21@163.com

✉ Ming Chen
chenming@xinhuamed.com.cn

[1] Department of Neurosurgery, Xinhua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, 1665 Kong Jiang Road, Yangpu District, Shanghai 200092, China

[2] Department of Emergency and Critical Care Medicine, Kong Jiang Hospital of Yangpu District, Shanghai 200082, China

[3] Department of Plastic Surgery, Xuzhou Medical University Affiliated Xuzhou City Hospital, Xuzhou 221000, Jiangsu, China

[4] Department of Neurosurgery, The First Affiliated Hospital of USTC, Division of Life Sciences and Medicine, University of Science and Technology of China, 17 Lu Jiang Road, Luyang District, Hefei 230001, Anhui, China