HOTAIR upregulates an 18-gene cell cycle-related mRNA network in glioma

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Abstract. HOTAIR is a tumor promoting long non-coding RNA (lncRNA) with roles in multiple cancers. However, the role of HOTAIR in glioma has not been well charaterized. Genes that positively correlated with HOTAIR were identified from the Chinese Glioma Genome Atlas and constructed into an interacting network. In total, 18 genes with P-values <0.01 were further extracted and constructed into a subnetwork. Real-time PCR, western blot and immunofluorescence analyses were employed to examine the expression of the genes after HOTAIR overexpression or knockdown. Intracranial glioblastoma multiform (GBM) models were used to test the potential of HOTAIR as a glioma therapy target. It was discovered that the 18 genes that most significantly correlated with HOTAIR expression formed a cell cycle-related mRNA network, which is positively regulated by HOTAIR. Furthermore, HOTAIR knockdown inhibited mouse intracranial GBM model formation. HOTAIR positively regulates a cell cycle-related mRNA network in glioma, and could be a potential therapeutic target for treating glioma.

Introduction

Gliomas are the most common primary brain tumors. The average survival rate of grade III patients is 3-5 years, whereas for GBM patients, the average survival is 12-15 months. Surgical resection, standard chemotherapy and radiation therapy provide little improvement in the outcomes of these patients (1). Thus, further exploration of the molecular mechanism may shed light on the treatment of glioma.

Long non-coding RNAs (lncRNAs) are a type of non-coding RNAs that are longer that 200 nt, and play various roles in diverse biological processes. They can affect expression of downstream genes, alter alternative splicing by hybridizing to pre-mRNA, are involved in structural and organizational roles in the cell and can be processed into small RNAs (2-4). However, the molecular mechanisms of lncRNA are not completely understood.

HOTAIR is an lncRNA that is much more characterized than other long non-coding transcripts. In developmental processes, HOTAIR silences the expression of the *HOXD* gene cluster (5). Targeted disruption of HOTAIR leads to gene de-repression and homeotic transformation in mice (6). Deletion of 5' *HOXC* genes where *HOTAIR* locates, leads to malformations in mice, such as clubfoot and vertical talus (7). HOTAIR was first reported as a tumor-promoting lncRNA in breast cancer (8). Increased research indicates that HOTAIR could be a potential biomarker and target in gastric cancer, colon cancer, cervical cancer and glioma (9-12). Mechanistically, the 5' domain of HOTAIR binds to the PRC2 complex, while the 3' domain binds to the LSD1 complex. EZH2 is a major component of the PRC2 complex, which tri-methylates H3K27 markers at the promoter of target genes. High levels of HOTAIR in cancer may inhibit tumor suppressor genes in epigenetic manner (13). Knocking down HOTAIR in glioma has been shown to upregulate NLK, a negative regulator of β-catenin pathway, which depends on the 5' domain but not the 3' domain (14). As a cell cycle-associated gene, HOTAIR is a strong predictor of survival in glioma, and is often expressed in classical and mesenchymal subtypes (15).

Accurate control of the cell cycle is essential for DNA synthesis and cell proliferation. Aberrant cell cycle progression is commonly observed in tumors and closely tracks with abnormal expression of cell cycle checkpoint genes. LncRNAs impact the cell cycle in various ways (16-18). $NcRNA_{CCND1}$ is a transcriptional regulator that suppresses cyclin D1 and participates in G1 arrest in a DNA damage-dependent manner (19). ANRIL, an antisense lncRNA in the INK4 locus, suppresses $p15^{INK4B}$ expression in a PRC2-dependent manner (20). MALAT1, a nuclear-localized lncRNA, controls the cell cycle

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via regulating B-MYB (21). Previously, we reported HOTAIR as a cell cycle-associated lncRNA. Herein, we further characterized the regulation mechanisms of HOTAIR.

In the present study, we identified a cell cycle-related mRNA network that is regulated by HOTAIR in glioma cells. Genes in the CGGA database whose expression is positively correlated with HOTAIR were constructed into an interaction network, ranked by connection. The top 18 genes with P-values <0.01 are associated with the cell cycle. In glioma cell lines, HOTAIR upregulated the mRNA expression of network, whereas knocking down HOTAIR inhibited expression of these genes. Finally, we demonstrated that knocking down HOTAIR in U87vIII cells significantly inhibited intracranial tumor growth. These results support the potential of targeting HOTAIR in glioma, and further supported that HOTAIR is the potential therapy target in glioma.

Materials and methods

Datasets of glioma samples. mRNA expression datasets and the corresponding clinical information were downloaded from the following websites: Chinese Glioma Genome Atlas (CGGA) (http://www.cgga.org.cn), the Cancer Genome Atlas (TCGA) (http://cancergenome.nih.gov), and the REpository for Molecular BRAin Neoplasia DaTa (REMBRANDT) (http://caintegrator.nci.nih.gov/rembrandt/).

Cell line and culture conditions. The human GBM cell lines U87, U87vIII and U251 were used for experiments. U87 and U251 GBM cells were purchased from the ATCC (Manassas, VA, USA). U87 cells were cultured in complete MEM medium, while U251 cells were cultured in complete EMEM medium containing 10% FBS, and incubated at 37˚C, 5% CO_2. U87vlll cells were stably transfected with a truncated mutant EGFR gene, which can be consistently activated without EGF stimulation. Puromycin (100 ng/ml) was added into U87vIII culture medium to maintain the stability of EGFRvIII.

Reverse transcription (RT)-PCR. To determine mRNA expression, after treatment, the glioma cells were lysed in TRIzol reagent (Sigma-Aldrich, St. Louis, MO, USA). The lysate was well mixed with chloroform and spun for 15 min at 13,000 x g at 4˚C. The top aqueous phase, which contains RNA, was collected and mixed with propan-2-ol to precipitate the total RNA, which was used for real-time PCR analysis of mRNA. The cDNA was synthesized from 1 μg of total RNA, using a reverse transcription kit purchased from Promega (Madison, WI, USA), following the manufacturer's protocol. Real-time PCR was performed using a SYBR Green Master Mix from Life Technologies (Carlsbad, CA, USA). Amplification was performed by the DNA Engine Opticon 2 Two-Color Real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA). Relative gene expression was analyzed by 2ΔΔ-Cq method (22). Gene specific primers: HOTAIR-F: ATAGGC AAATGTCAGAGGGTT, HOTAIR-R: TCTTAAATTGG GCTGGGTC; GAPDH-F: GGTGGTCTCCTCTGACT TCAACA, GAPDH-R: GTTGCTGTAGCCAAATTCGT TGT; CCNA2-F: CTCTACACAGTCACGGGACAAAG, CCNA2-R: CTGTGGTGCTTTGAGGTAGGTC; HMMR-F: GGCTGGGAAAAATGCAGAGGATG, HMMR-R: CCTTT AGTGCTGACTTGGTCTGC; FoxM1-F: CCTTCTGGA CCATTCACCCC, FoxM1-R: TCACCGGGAACTGGATA GGT; NUSAP1-F: CTGACCAAGACTCCAGCCAGAA, NUSAP1-R: GAGTCTGCGTTGCCTCAGTTGT; ASPM-F: GAGACCTTGGTGGAATACCTGC, ASPM-R: ACGAAGA TCCAAAAGCCTTGCAC; CDC6-F: TCCACCAAAGCAA GGCAAGA, CDC6-R: CGATCTGGGACAGCTGTGTT; FANCI-F: GCAAGCTGATGTTCGACTCATGC, FANCI-R: AGGCAGCAGATCAGGTTTTGGC; NCAPG-F: GACGA ACAGGAGGTGTCAGACT, NCAPG-R: TGCTGCGGT TTTGGCTCGTCTT; DLGAP5-F: CTCGATCAGCTACT CAAGCAGC, DLGAP5-R: CAGGTCTTCCTTTACTTG GCACC; CHEK1-F: ATCAACTCATGGCAGGGGTG, CHEK1-R: TCCAGCGAGCATTGCAGTAA; CEP55-F: TCG ACCGTCAACATGTGCAGCA, CEP55-R: GGCTCTGTG ATGGCAAACTCATG; KIF4A-F: TGCGTGGTCAAGTTT CGGAGTC, KIF4A-R: GCTGTAGGTCAGCAATCTGAGC; HJURP-F: TGAGAATTTGGGGTGGAAGACT, HJURP-R: AGCGGAGTCACACGTACATC; PLK4-F: GACACCTCAG ACTGAAACCGTAC, PLK4-R: GTCCTTCTGCAAATC TGGATGGC; CCNB2-F: AGTTCCAGTTCAACCCACCAA, CCNB2-R: TTGCAGAGCAAGGCATCAGA; CENPE-F: GGAGAAAGATGACCTACAGAGGC, CENPE-R: AGTT CCTCTTCAGTTTCCAGGTG; NCAPH-F: GACGAACAG GAGGTGTCAGACT, NCAPH-R: TGCTGCGGTTTTGG CTCGTCTT; AURKB-F: CATCCCAACATCCTGCGTCT, AURKB-R: AGCTCTCCCTTGAGCCCTAA.

Western blot analysis. Protein lysates were prepared as follows: after treatment, cells were washed twice with cold PBS, scraped and lysed in ice-cold RIPA buffer (Solarbio, Beijing, China). Lysate was sonicated for 20 cycles, and microcentrifuged for 15 min at 4˚C. The supernatant was transferred to a new test tube and stored at 20˚C. The protein samples were resolved by SDS-PAGE and transferred onto PVDF membranes (Milipore, Darmstadt, Germany). The membranes were then incubated with the following antibodies: anti-FoxM1 (Cell Signaling Technology, Danvers, MA, USA), anti-CEP55 (Abcam, Cambridge, UK) and GAPDH (Proteintech, Wuhan, China). Antibody-labeled protein bands on the PVDF membranes were detected using a G:BOX F3 (Syngene, Cambridge, UK).

Immunofluorescence analysis. U87 cells were seeded onto the coverslips and transfected with negative control or Lenti-HOTAIR virus (GeneChem, Shanghai, China) for 48 h. Then, they were fixed in 4% paraformaldehyde for 30 min. The cells or sections were permeabilized with 0.1% Triton-X 100 for 10 min, followed by blocking with 3% BSA for 1 h in RT. Immunofluorescence staining was conducted with antibodies against CEP55 (Abcam, 1:100) and CENPE (Abcam, 1:100). The cells were washed with PBS and incubated with Alexa Fluor 633 or Alexa Fluor 594 (Life Technologies) secondary antibodies. Nuclei were stained using DAPI and the cells were visualized using FV-1200 laser scanning confocal microscope.

Mouse glioma intracranial model and treatment. U87vIII cells were transfected with either a negative control or Lenti-si-HOTAIR virus. An indicated number of cells in suspension were injected stereotactically into the brain of 4-week-old BALB/c-nu mice. The mice were sacrificed at day 14.

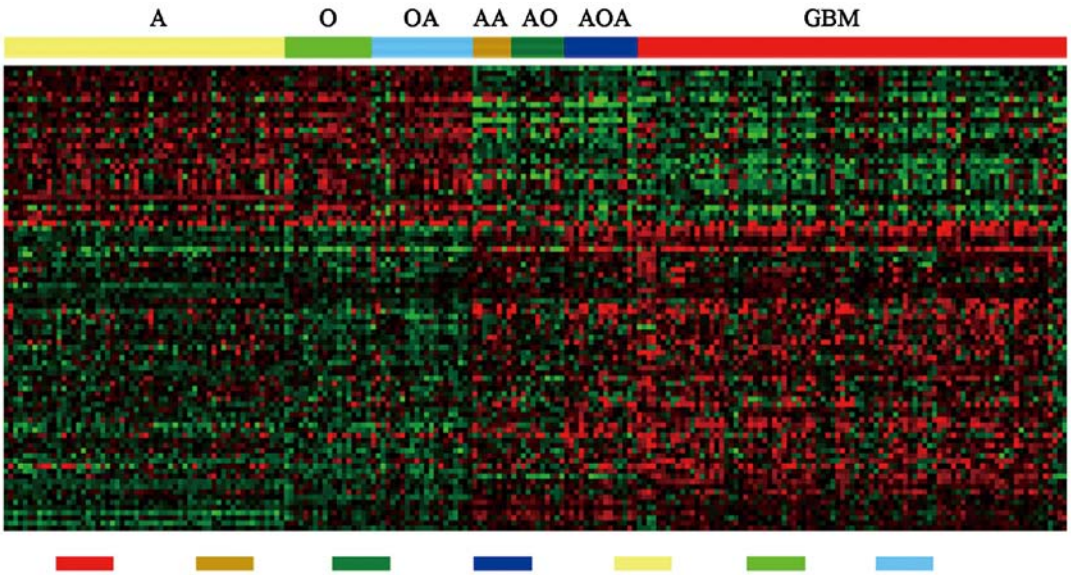

Figure 1. Differing patterns of lncRNA expression in glioma. The relative expression of lncRNAs in CGGA database was sorted by the clinical glioma grades.

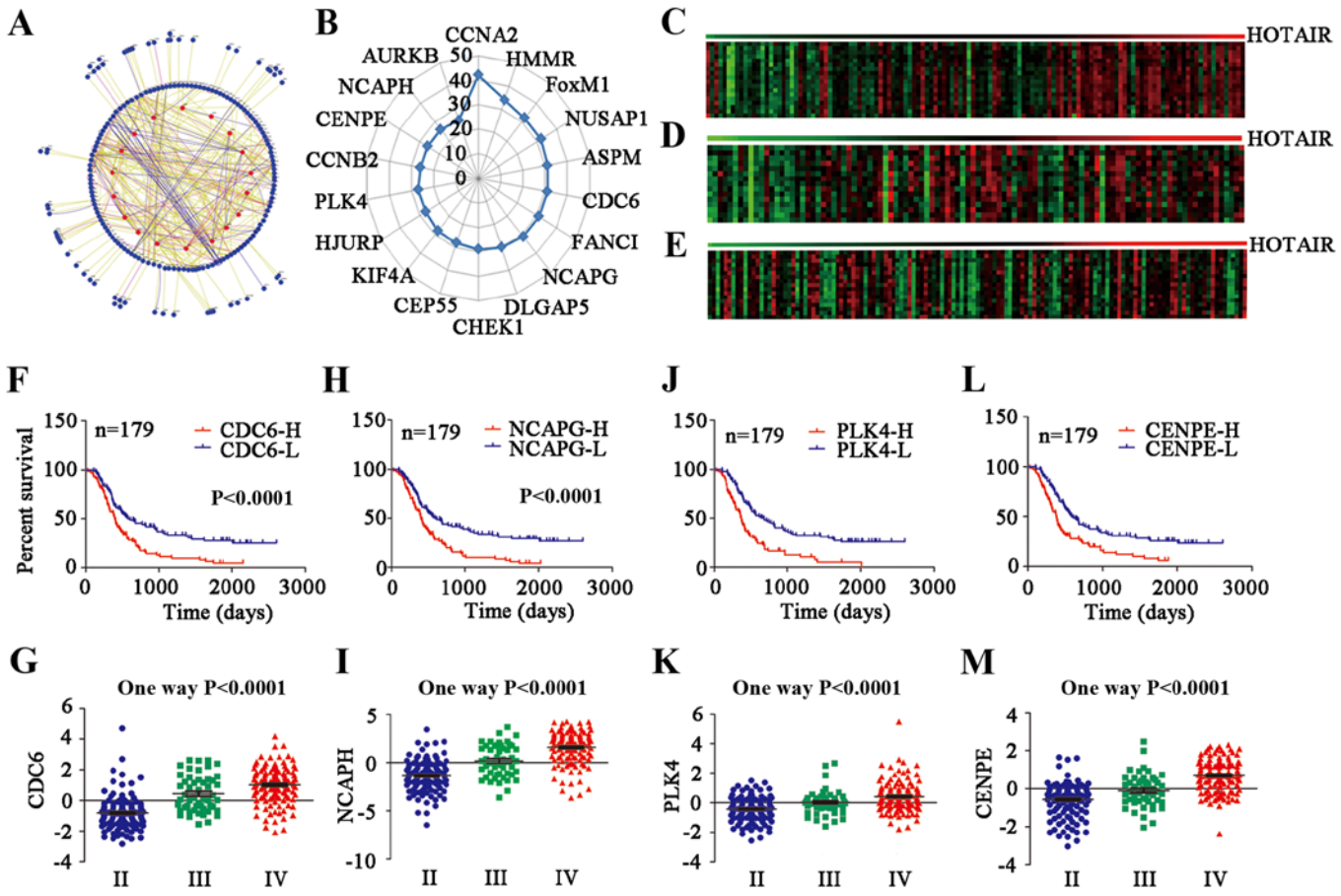

Figure 2. HOTAIR correlates with a cell cycle-related mRNA network. (A) Genes positively correlated with HOTAIR were constructed into an interacting network. (B) Genes with P-values <0.01 were selected and incorporated into a subnetwork based on connection. These 18 genes were clustered according to HOTAIR expression in the CGGA (C), TCGA (D) and REMBRANT databases (E). The expression of CDC6 (F), NCAPH (H), PLK4 (J) and CENPE (L) in different WHO grades was analyzed with the CGGA database. According to the expression value of CDC6 (G), NCAPH (I), PLK4 (K) and CENPE (M), patients with high-grade glioma could be divided into two groups with significantly different prognoses in the CGGA database. High group: patients with indicated gene expression higher than or equal to the median one. Low group: patients with lower indicated gene levels lower than the median one.

Histological analysis. The xenograft samples were collected at day 14 after tumor implantation, and were subjected to Hematoxylin and eosin (H&E) staining. Sections (5 μm) were cut, dehydrated, deparaffinized, and rehydrated.

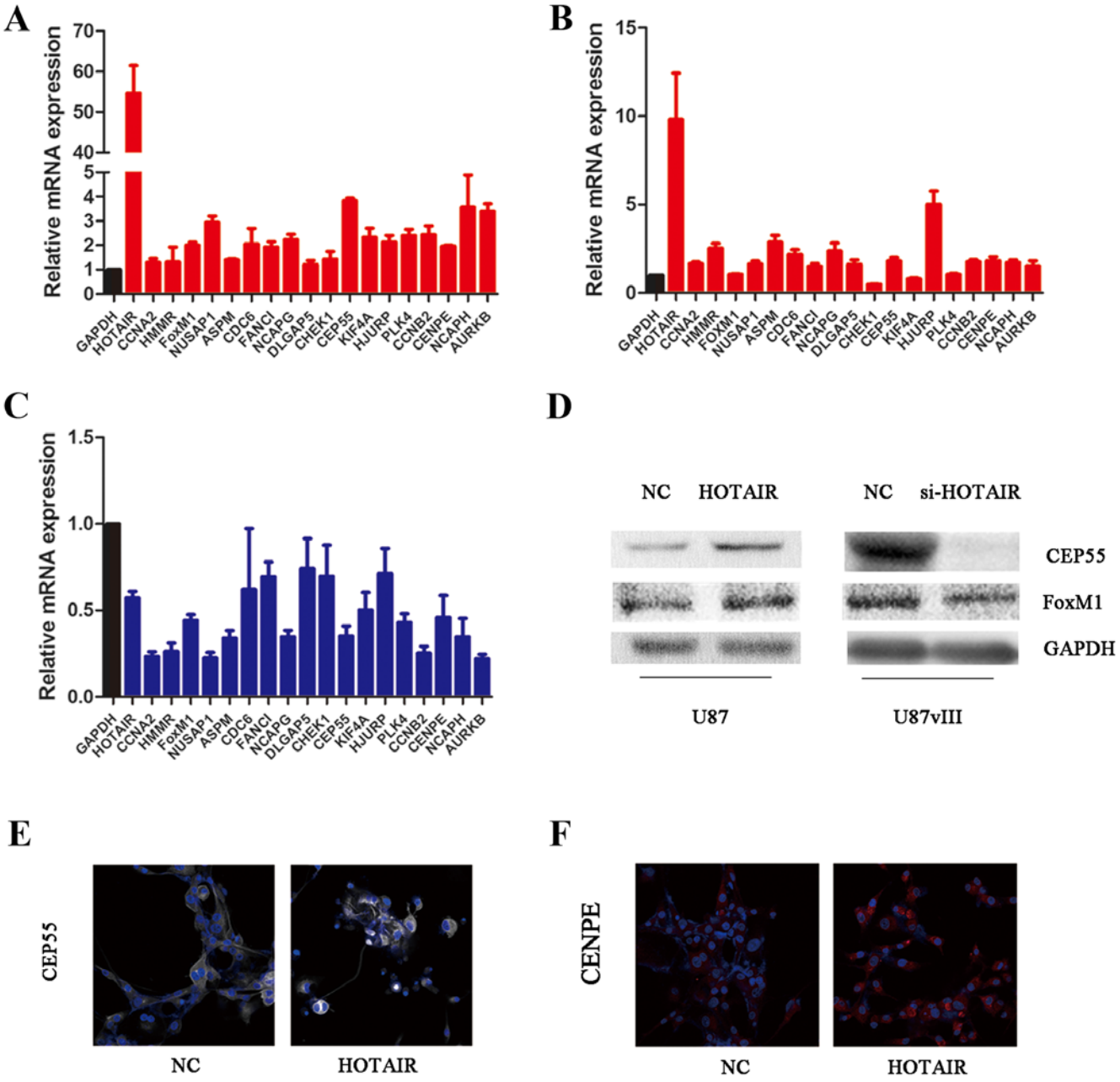

Figure 3. HOTAIR positively regulates the cell cycle-related mRNA network. The expression of 18 genes in the cell cycle-related mRNA network was assessed by real-time PCR after HOTAIR overexpression in U87 (A) and U251 cells (B), and HOTAIR knockdown in U87vIII cells (C). (D) The expression of FoxM1 and CEP55 was examined by western blotting in U87 and U87vIII cells, with overexpression or knockdown of HOTAIR. CEP55 (E) and CENPE (F) were examined by immunofluorescence after HOTAIR overexpression in U87 cells.

H&E staining was performed according to the standard protocols. All images were captured via microscopy (Olympus, Tokyo, Japan). The protocol for the animal study was approved by the Animal Ethics Committee of Tianjin Medical University.

Statistical analysis. Statistical analysis was performed using the SPSS Graduate Pack, version 11.0, statistical software (SPSS). Data are presented as means ± SEM of three independent experiments or means ± SD performed in triplicate. One-way ANOVA was used for comparison among the different groups. A P-value of 0.05 was considered to indicate a statistically significant difference.

Results

Identification of a cell cycle-related mRNA network positively correlated with HOTAIR. Although variety of lncRNAs have been described in recent years, the functions of lncRNA are still not completely characterized. To profile lncRNAs in glioma, cluster analysis was employed using the CGGA database, which includes 220 glioma samples and 5 normal brain samples. There are 90 lncRNAs that have different patterns of expression between low-grade and high-grade gliomas. There are 31 lncRNAs downregulated in high-grade glioma, while 59 lncRNAs were upregulated (FDR <0.05, fold >1.5) (Fig. 1). It is possible that the main alteration of lncRNA expression

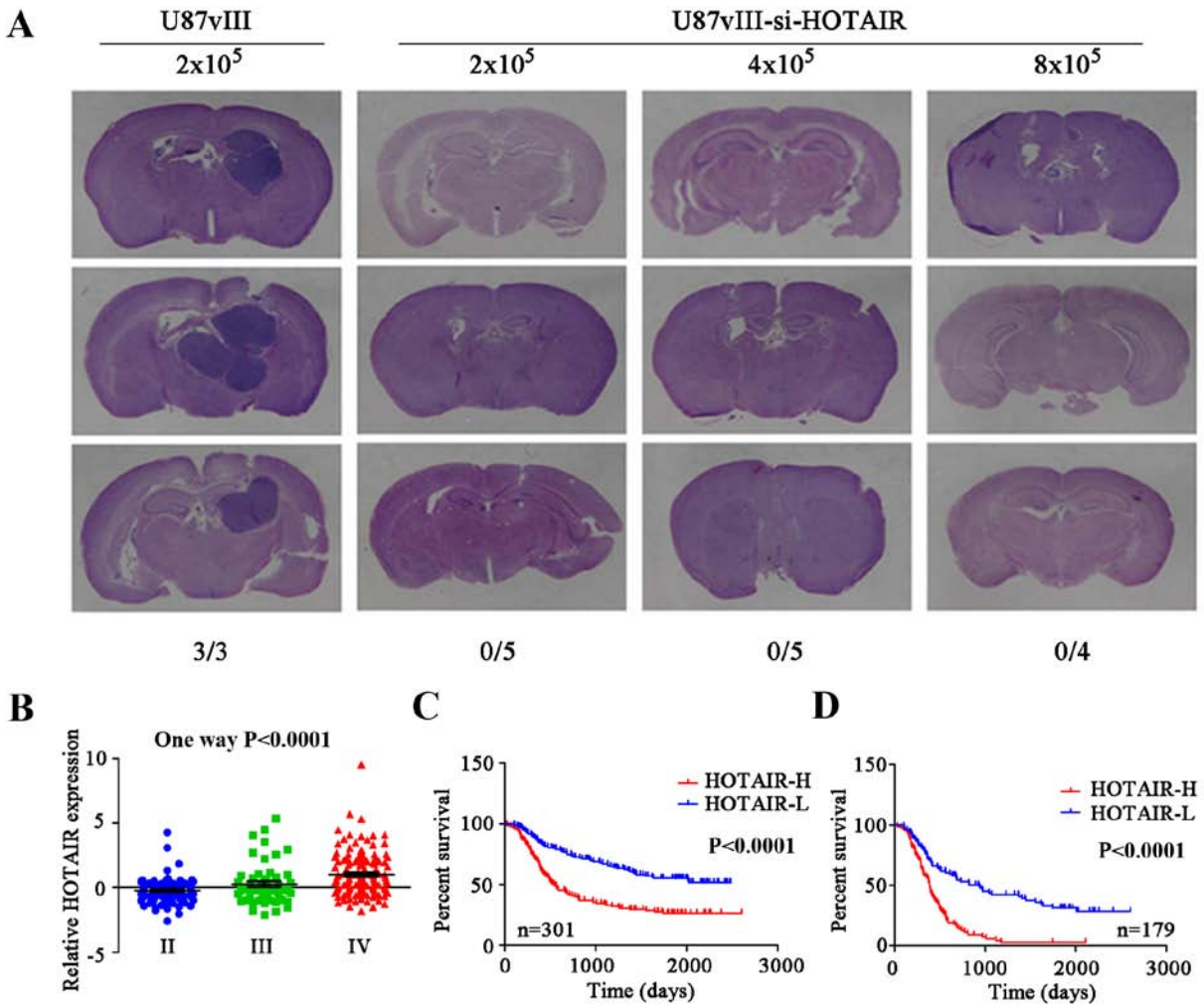

Figure 4. Targeting HOTAIR inhibits U87vIII intracranial glioma model. (A) H&E staining of tissue from mice with U87vIII (negative control or Lenti-si HOTAIR treatment) on day 14 after implantation. (B) HOTAIR expression levels in WHO glioma grades were analyzed in the CGGA database. Kaplan-Meier survival curves for HOTAIR expression in glioma (C) and Grade III and IV (D).

occurs during malignant progression from low-grade glioma to GBM, indicating an important role of lncRNA in glioma progression. HOTAIR is among the lncRNAs in the high expressin module of the heat map, which is consistent with a previous study (15). HOTAIR is a cell cycle-associated oncogene in glioma (11), however, further research is still needed to demonstrate its role in gliomagenesis.

To profile the function of HOTAIR, genes positively correlated with HOTAIR were selected by Pearson correlation coefficients (R>0.3, P<0.05) (Fig. 2A). In total, 244 HOTAIR correlated genes formed a complex network, in which they correlated with each other according to literature, databases or experiments. Genes with P-values <0.01 were extracted from the HOTAIR positive gene network, as they are more significantly correlated with HOTAIR (Fig. 2B). The correlation degrees of the 18 genes are greater than 25, and all of them interact with each other. Overall, 215 of the 244 genes in network are directly correlated with these 18 genes, implying a key role of this 18-gene network. The cluster analysis of these genes according to HOTAIR expression in the CGGA, REMBRANDT and TCGA databases further confirmed the positive correlation expression pattern of these 18 genes with HOTAIR (Fig. 2C-E). Bioinformatic analysis revealed that the upregulation of 17 of them (except CCNB2) corresponds with clinical stage glioma (Table I). Kaplan-Meier survival analysis further indicated a negative correlation between the expression level of 17 genes and the survival rate in high-grade glioma. Fig. 2F-M depicts four genes with the most significant P-values from the Kaplan-Meier survival analysis. Of these 18 genes, 15 genes play roles in cell cycle regulation, including CCNA2, FoxM1, CEP55, CENPE. FANCI and CDC6 are genes regulating DNA replication and repair, which may influence the cell cycle indirectly. Thus, we identified these mRNAs as a cell cycle-related network that positively correlated with HOTAIR.

HOTAIR upregulates a cell cycle-related mRNA network in vitro. To further test if this mRNA network could be upregulated by HOTAIR *in vitro*, we transfected a tetracycline-inducible HOTAIR expression lenti virus. Real-time PCR analysis indicated that genes in this network were upregulated when HOTAIR expression was induced by doxycycline in U87 (Fig. 3A) and U251 (Fig. 3B) cells. EGFRvIII is an aggressive EGFR mutation type in glioma. We knocked down HOTAIR in U87vIII cells by Lenti-si-HOTAIR and discovered that this network was downregulated by inhibiting HOTAIR (Fig. 3C).

Table I. The cell cycle-related mRNA network that positively correlated with HOTAIR.

Genes	Degrees	Survival in HGG (P-value)	UniProtKB/Swiss-Prot
CCNA2	42	0.0004	Controlling the cell cycle at the G1/S (start) and the G2/M (mitosis) transitions
HMMR	34	0.0018	Phosphorylation of a number of proteins, including PTK2/FAK1
FoxM1	32	0.0032	Regulates the expression of cell cycle genes essential for DNA replication and mitosis
NUSAP1	32	0.0011	Organization of mitotic spindle microtubules around them
ASPM	31	0.0002	Mitotic spindle regulation and coordination of mitotic processes
CDC6	31	<0.0001	Initiation of DNA replication
FANCI	31	0.0001	Repairing of DNA double-strand breaks by homologous recombination
NCAPG	31	<0.0001	Conversion of interphase chromatin into mitotic-like condense chromosomes
DLGAP5	30	0.0026	Cell cycle regulator and key regulator of adherens junction integrity
CHEK1	29	0.0059	Binding to and phosphorylating CDC25A, CDC25B and CDC25C
CEP55	28	0.0016	Mitotic exit and cytokinesis
KIF4A	28	0.0117	Mitotic chromosomal positioning and bipolar spindle stabilization
HJURP	27	0.0334	Incorporation and maintenance of histone H3-like variant CENPA at centromeres
PLK4	27	<0.0001	Central role in centriole duplication
CCNB2	26	0.0646	Controlling the cell cycle at the G2/M (mitosis) transition
CENPE	26	<0.0001	Maintenance of chromosomal stability
NCAPH	26	0.0032	Regulatory submit of the condensing complex
AURKB	25	0.0077	Key regulator of mitosis

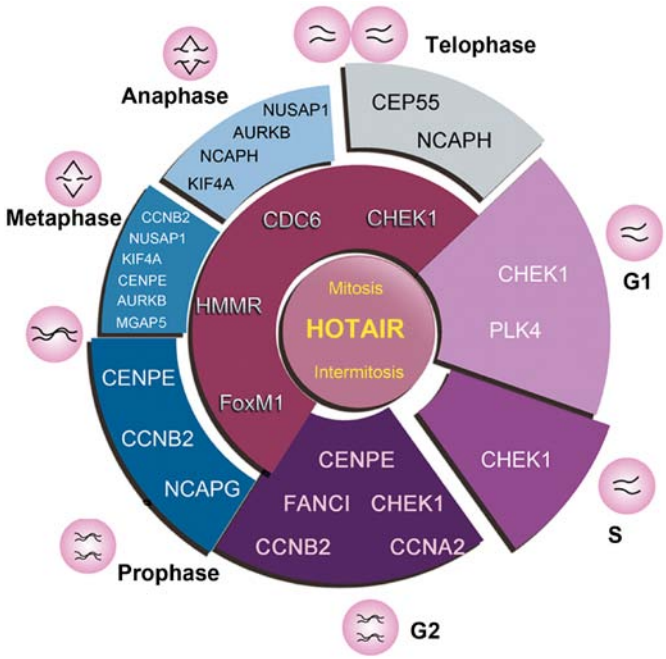

Figure 5. Genes in HOTAIR upregulated mRNA network are distributed throughout the cell cycle.

FoxM1 is a key transcription factor that regulates the expression of cell cycle genes (23,24). CEP55 and CENPE, which are important regulators of mitosis, impact survival rate very significantly in high-grade glioma (HGG) (24,25). Thus, we representatively chose FoxM1, CEP55 and CENPE from the network to further examine the influence of HOTAIR on these genes and the proteins they code. Western blot analysis indicated that HOTAIR could upregulate FoxM1 and CEP55 in U87 cells, while Lenti-si-HOTAIR-treatment inhibited expression of these proteins in U87vIII cells (Fig. 3D). Immunofluorescence staining further confirmed the upregulation of CEP55 (Fig. 3E) and CENPE (Fig. 3F) in U87 cells. Thus, we demonstrated that this cell cycle-related mRNA network is positively regulated by HOTAIR.

Targeting HOTAIR inhibits glioma progression. EGFR is a receptor tyrosine kinase that is frequently amplified and mutated in several cancers. EGFRvIII is one such mutation commonly found in GBM patients, and it contributes to the malignant progression of the disease (26). Knocking down HOTAIR significantly inhibited the cell cycle-related mRNA network in U87vIII cells. Thus, we targeted HOTAIR in U87vIII intracranial GBM mouse models. U87vIII cells ($2x10^5$) could form orthotopic tumors in mice in 14 days. However, after lenti-si-HOTAIR treatment, there was no macroscopic tumor formation even after the injection of $8x10^5$ cells (Fig. 4A). These results indicated that knocking down HOTAIR could control U87vIII tumor formation within 2 weeks. Our bioinformatic analysis of the CGGA database further indicated that HOTAIR expression is upregulated in high-grade glioma (Fig. 4B), and high levels of HOTAIR are correlated with poor outcome in glioma (Fig. 4C) and HGG (Fig. 4D). These results indicated that HOTAIR could be a potential therapeutic target in glioma, especially for GBM.

Discussion

In the present study, we demonstrated that HOTAIR regulates a cell cycle-related mRNA network in glioma. Genes in this

network are distributed throughout the cell cycle (Fig. 5). When leaving G_0 phase to enter into mitosis, healthy cells check the integrity of chromosomes and proteins required for replication. CCNA2, CCNB2 and CHEK1 promote this cell cycle checkpoint (27-29). During mitosis, the localization of centrosomes and spindles is well organized to perform accurate separation of the two daughter cells. NCAPH, CENPE, KIF4A, CEP55, NUSAP1 and ASPM participate in mitotic spindle regulation and mitotic processes (25,30-34). FoxM1 and AURKB are key regulators of mitosis (35-37). Thus, HOTAIR may regulate the cell cycle in glioma by impacting both checkpoint proteins and functional proteins during multiple mitosis steps.

Ten genes [HMMR (38), FoxM1 (39), ASPM (40), CDC6 (41), NCAPG (42), CHEK1 (43), CEP55 (44), HJURP (45), CENPE (42), ARUKB (46)] in this network are reported to promote glioma, through regulating the cell cycle and chemoresistance in glioma. Although the other genes have not yet been deeply studied in glioma, their expression is positively correlated with clinical grade glioma (Table I), which makes them potential therapeutic targets. Moreover, they participate in tumor progression in several cancers, including lung cancer and breast cancer (47-52). This evidence further confirmed the tumor-promoting role of HOTAIR in glioma and other cancers. Our results indicated that HOTAIR performs its carcinogenesis effort not only by inhibiting tumor-suppressor genes, but also by promoting expression of various oncogenes. This is the first report that HOTAIR could positively regulate a complex oncogene mRNA network, which further profiled the function of HOTAIR. However, the mechanism of upregulation of this network by HOTAIR remains to be explored. This upregulation could either be directly executed by HOTAIR, or be the cascading effort of network interactions.

In conclusion, we report for the first time that HOTAIR could positively regulate a complex oncogene mRNA network, which contributes to the further characterization of the functions of HOTAIR in glioma.

Acknowledgements

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