High expression of LncRNA HOTAIR is a risk factor for temozolomide resistance in glioblastoma via activation of the miR-214/β-catenin/MGMT pathway

Tian Lan
2019203030036@whu.edu.cn
Zhongnan Hospital of Wuhan University

Wei Quan
2016302180065@whu.edu.cn
Zhongnan Hospital of Wuhan University

Dong-Hu Yu
donghu_yu@whu.edu.cn
Zhongnan Hospital of Wuhan University

Xi Chen
2018305230073@whu.edu.cn
Zhongnan Hospital of Wuhan University

Ze-Fen Wang
wangzf@whu.edu.cn
Wuhan University

Zhi-Qiang Li
lizhiqiang@whu.edu.cn
Zhongnan Hospital of Wuhan University

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High expression of LncRNA HOTAIR is a risk factor for temozolomide resistance in glioblastoma via activation of the miR-214/β-catenin/MGMT pathway

Tian Lan[1],Wei Quan[1], Dong-Hu Yu[1], Xi Chen[1], Ze-Fen Wang[2]*, Zhi-Qiang Li[1]*

[1]Department of Neurosurgery, Zhongnan Hospital of Wuhan University, Wuhan, Hubei, China

[2]Department of Physiology, Wuhan University School of Basic Medical Sciences, Wuhan, Hubei, China

* **Corresponding author**

Zhi-Qiang Li, E-mail addresses: lizhiqiang@whu.edu.cn

Ze-Fen Wang, E-mail addresses: wangzf@whu.edu.cn

Abstract

Backgroud: HOX transcript antisense RNA (HOTAIR) is upregulated in glioblastoma (GBM) and associated with temozolomide (TMZ) resistance. However, the mechanisms underlying HOTAIR-mediated TMZ resistance remains poorly understood.

Methods: HOTAIR expression in glioma-related public datasets and drug response estimation were analyzed using bioinformatics. These findings were verified by overexpressing HOTAIR in TMZ-sensitive U251 cells and/or silencing HOTAIR in resistant U251 cells (U251R). The cytotoxic effects were evaluated using cell viability assay and flow cytometry analysis of cell clycle and apoptosis.

Results: HOTAIR was upregulated in TMZ-resistant GBM cell lines and patients with high HOTAIR expression responded poorly to TMZ therapy. HOTAIR knockdown restored TMZ sensitivity in U251R cells, while HOTAIR overexpression conferred TMZ resistance in U251 cells. Wnt/β-catenin signaling was enriched in patients with high HOTAIR expression; consistently, HOTAIR positively regulated β-catenin expression in U251 cells. Moreover, HOTAIR-mediated TMZ resistance was associated with increased MGMT protein level, which resulted from the HOTAIR/miR-214-3p/β-catenin network.

GBM with high HOTAIR expression exhibited sensitivity to methotrexate. Methotrexate enhanced TMZ sensitivity in U251R cells, accompanied by reduced expression of HOTAIR and β-catenin. **Conclusions:** HOTAIR is a risk factor for TMZ resistance and methotrexate may represent a potential therapeutic drug for patients with high HOTAIR expression level.

Keywords: lncRNA HOTAIR; temozolomide; resistance; β-catenin; methotrexate

Introduction

Glioblastoma (GBM) is an aggressive form of glioma characterized by a high recurrence rate and poor prognosis. Since a randomized phase III trial in 2005 reported an improved survival rate with radiotherapy plus temozolomide (TMZ) [1, 2], the combined use of TMZ and radiotherapy has become the standard postoperative treatment for patients with GBM [3]. However, this combined regimen yielded only a modest improvement in the median survival, from 12.1 to 14.6 months [2], benefiting only approximately 14% of newly diagnosed patients who received TMZ [4]. This limited survival benefit is largely caused by the emergence of inherent and acquired TMZ resistance, which not only leads to tumor recurrence but is also a major obstacle in the long-term management of patients with GBM [5, 6]. Thus, it is crucial to explore the intrinsic factors

affecting the TMZ response and develop strategies to improve the efficacy of TMZ chemotherapy.

Long noncoding RNAs (lncRNAs) are functional RNA molecules exceeding 200 nucleotides. LncRNAs do not encode proteins but exert regulatory control at the epigenetic, transcriptional, and post-transcriptional levels. They play critical roles in various biological processes in tumors, including proliferation, invasion, angiogenesis, and chemoresistance [7, 8]. Research indicates that aberrant lncRNA expression is common in tumors, and some may serve as potential biomarkers for tumor diagnosis and prognosis [9-12]. HOX transcript antisense RNA (HOTAIR) is a widely studied oncogenic lncRNA. HOTAIR, nearly absent in normal brain tissue, is upregulated in human gliomas [13] and TMZ-resistant GBM cells [14]. Serum-derived exosomal HOTAIR induces TMZ resistance in GBM cells, whereas HOTAIR depletion enhances TMZ sensitivity both in vivo and in vitro [15]. However, the precise molecular mechanisms underlying HOTAIR-mediated TMZ resistance remain poorly understood.

In this study, we investigated HOTAIR expression in TMZ-resistant CBM cell lines and explored the associated signaling pathways using bioinformatics. We observed the enrichment of the

Wnt/β-catenin pathway, a signaling pathway closely correlated with poor survival and TMZ resistance in gliomas [16], in patients with high HOTAIR expression. Next, we verified the association between HOTAIR and TMZ resistance by overexpressing HOTAIR in sensitive U251 GBM cells and silencing HOTAIR in resistant U251 cells (U251R). Subsequently, we explored the role of β-catenin signaling in HOTAIR-related TMZ resistance. Finally, we identified potential therapeutic compounds for patients with high HOTAIR expression levels. This study offers new insights into the predictive value of HOTAIR for TMZ sensitivity and suggests that HOTAIR could serve as a promising biomarker for individualized management of patients with GBM.

Results

HOTAIR is upregulated in TMZ-resistant GBM cells and patients with recurrent GBM

To identify the lncRNAs associated with acquired TMZ resistance in glioma, we first searched for differentially expressed lncRNAs in the datasets of TMZ-resistant GBM cell lines, U251(GSE100736) and LN299 (GSE113510), as well as in a cohort of three pairs of human primary and recurrent GBM samples (GSE7697). Because oncogenic lncRNAs are generally upregulated and tumor-suppressive lncRNAs

are frequently downregulated in tumors, we focused on upregulated lncRNAs in these datasets; 27, 75, and 5 lncRNAs were upregulated in the GSE100736, GSE113510, and GSE7697 datasets, respectively (Fig. 1A). HOTAIR was the only lncRNA that intersected the datasets (Fig. 1B), suggesting that HOTAIR may play a critical role in acquired TMZ resistance and GBM recurrence.

Molecular biomarkers, such as mutant isocitrate dehydrogenase 1(IDH1), MGMT promoter methylation, and the 1p19q co-deletion, are closely associated with the prognosis of patients with glioma and have been introduced into the WHO classification criteria for glioma [17]. By analyzing the data from TCGA, CGGA, and GSE16011 datasets, we found a significant correlation between HOTAIR expression and tumor grade and the status of IDH mutations, MGMT promoter methylation, and 1p19q co-deletion. Patients with GBM (grade 4) exhibited significantly higher HOTAIR expression than those with lower grades (grades 2 and 3; Fig. 1C). Patients with mutant IDH1, methylated MGMT promoter, or 1p19q co-deletion had lower HOTAIR expression than those with wild-type IDH1, unmethylated MGMT promoter, or non-co-deleted 1p19q (Fig. 1D). Moreover, patients with high HOTAIR expression had poorer overall survival than those with low HOTAIR expression (Fig. 1E). Similar

results were observed in patients receiving TMZ chemotherapy (Fig. 1F), indicating that patients with high HOTAIR expression had poorer responses to TMZ.

HOTAIR knockdown restores TMZ sensitivity in resistant GBM cells

To confirm the role of HOTAIR in the development of TMZ resistance, we developed a TMZ-resistant GBM cell line, U251R (Fig. 2A), and examined changes in HOTAIR expression. Consistent with our bioinformatics findings, U251R cells had higher HOTAIR expression levels than the parental cells (Fig. 2B). In U251R cells, silencing HOTAIR increased TMZ sensitivity compared to control siRNA (IC_{50}: 657.9±81.8 μM *vs.* 1057±113 μM, Fig. 2C-D). The cytotoxic effects of TMZ are mediated by a wide spectrum of methyl adducts that trigger G2/M cell cycle arrest and apoptosis [18, 19]. Compared to the control siRNA, HOTAIR silencing significantly increased TMZ-induced G2/M arrest (Fig. 2E) and apoptosis (Fig. 2F-G). These fingdings indicate that HOTAIR plays an important role in acquired TMZ resistance.

HOTAIR overexpression confers TMZ resistance in sensitive GBM cells

Next, we explored whether U251 cells with high HOTAIR expression exhibited inherent resistance to TMZ treatment. HOTAIR-overexpressing U251 cells had a higher IC_{50} than the vector control (834.7±69.35 μM *vs.* 530.3±81.8 μM, Fig. 2H-I). HOTAIR overexpression did not affect the cell cycle or apoptosis of U251 cells; however, it reduced TMZ-induced G2/M arrest (Fig. 2J) and cell apoptosis (Fig. 2K). These results indicate that high HOTAIR expression in GBM cells confers inherent TMZ resistance.

HOTAIR positively regulates the expression of MGMT

It is well established that MGMT plays a critical role in TMZ resistance and its expression is inversely correlated with TMZ sensitivity [20-22]. Next, we examined whether HOTAIR had an effect on MGMT expression. In U251R cells, HOTAIR silencing reduced MGMT mRNA and protein levels (Fig. 3A-B). Consistently, in U251 cells, HOTAIR overexpression increased MGMT mRNA and protein levels (Fig. 3C-D). These findings confirm that HOTAIR positively regulates MGMT expression. Therefore, HOTAIR-induced TMZ resistance was associated with MGMT upregulation.

Beta-catenin/MGMT pathway is involved in HOTAIR-mediated TMZ resistance

We further explored the signaling pathways involved in HOTAIR-induced TMZ resistance. Wnt/β-catenin signaling is one of the most active pathways in GBM; more importantly, increased Wnt/β-catenin signaling correlates with poor survival and TMZ resistance [16]. Pathway enrichment analysis showed that the Wnt/β-catenin signaling pathway was enriched in patients with high HOTAIR expression (Fig. 4A). Moreover, U251R cells had a higher β-catenin protein level than U251 cells (Fig. 4B). We further examined the association between HOTAIR and β-catenin by knocking down HOTAIR in U251R cells and overexpressing it in U251 cells. In U251R cells, downregulation of HOTAIR significantly reduced β-catenin in the whole cell lysate and cytoplasmic and nuclear fractions (Fig. 4C). In U251 cells, HOTAIR overexpression had the opposite effects (Fig. 4D). These findings indicate that β-catenin is a downstream effector of HOTAIR.

To verify the role of β-catenin in HOTAIR-mediated TMZ resistance, we treated HOTAIR-silenced U251R cells with a β-catenin agonist, SKL2001, which inhibits the phosphorylation and degradation of β-catenin [23]. Compared to vehicle control, SKL2001 treatment significantly increased the total and nuclear β-catenin protein and MGMT protein in HOTAIR-silenced U251R cells (Fig. 4E-F),

supporting the regulatory role of β-catenin in MGMT expression. SKL2001 pre-treatment also reduced the TMZ-induced G2/M cell cycle arrest and apoptosis (Fig. 4G-H). Collectively, these results imply that β-catenin is involved in HOTAIR-mediated TMZ resistance by upregulating MGMT expression.

HOTAIR regulates the β-catenin/MGMT pathway by sponging miR-214-3p

Many lncRNAs act as competitive endogenous RNA (ceRNA) to regulate the expression of downstream target genes by sponging microRNAs [24]. miR-214 is a molecular hub that controls the signaling networks in tumors and directly targets the 3′-untranslated region of β-catenin mRNA to suppress its expression [25, 26]. HOTAIR modulates cell proliferation and invasion by sponging miR-214 in ovarian [27], lung [28], cervical [29], and colorectal cancer [30]. Therefore, we examined the role of miR-214 in HOTAIR-mediated TMZ resistance. U251R cells exhibited lower miR-214 levels than U251 cells (Fig. 5A). Silencing HOTAIR in U251R cells increased miR-214 expression, whereas HOTAIR overexpression in U251 cells decreased miR-214 expression (Fig. 5B). The inverse correlation between HOTAIR and miR-214 expression suggests that HOTAIR acts as a ceRNA to sponge miR-214. In U251R cells, miR-

214-3p mimics increased TMZ-induced G2/M arrest and apoptosis (Fig. 5C-D) and decreased β-catenin and MGMT protein levels (Fig. 5E). These results indicate that HOTAIR activates the β-catenin/MGMT pathway by binding to miR-214-3p.

MTX increases TMZ sensitivity in resistant GBM cells

To identify candidate drugs for patients exhibiting high HOTAIR expression, we assessed drug responses using two pharmacogenomic datasets (CTRP and PRISM) for each TCGA sample. As shown in Figure 6A, patients with high HOTAIR expression showed sensitivity to four CTRP-derived drugs (paclitaxel (PTX), methotrexate (MTX), brefeldin A, and SB-743291) and four PRISM-derived drugs (CYT-997, VE-822, LY2603618, and ADL5859).

Among these compounds, both PTX and MTX have received FDA approval for clinical use. MTX has limited blood-brain barrier (BBB) penetration at low doses, but reaches the central nervous system (CNS) effectively and shows efficacy against brain metastases and primary CNS lymphoma when administered at high doses [31, 32]. We further examined whether MTX could induce cytotoxicity in TMZ-resistant GBM cells. Our results demonstrated a significant reduction in the viability of the U251R cells in response to MTX treatment (Fig. 6B). MTX pre-treatment reduced the IC_{50} of TMZ in

U251R cells (Fig. 6C), indicating an increase in TMZ sensitivity. In U251R cells, MTX not only triggered G1 phase arrest and apoptosis (Fig. 6D-E) but also reduced HOTAIR mRNA and β-catenin protein levels (Fig. 6F-G). These results indicate that MTX enhances TMZ sensitivity, at least partially, by downregulating the HOTAIR/β-catenin pathway. Therefore, MTX is a potential therapeutic agent for the treatment of TMZ-resistant GBM.

Discussion

TMZ is a first-line chemotherapeutic agent for patients with GBM; however, inherent and acquired TMZ resistance pose major challenges to GBM treatment. Abnormal lncRNA expression mediates tumor chemoresistance [33]. In this study, we found that HOTAIR was upregulated in TMZ-resistant GBM cells, and patients with high HOTAIR expression had a poorer response to TMZ treatment than those with low HOTAIR expression. Further, HOTAIR knockdown restored TMZ sensitivity in U251R cells, and HOTAIR overexpression conferred TMZ resistance in U251 cells. These results point to high HOTAIR expression being a risk factor for inherent and acquired TMZ resistance and that HOTAIR likely serves as a predictive biomarker for TMZ sensitivity. Therefore, the

detection of HOTAIR expression in primary and recurrent tumors may guide the individualized management of patients with GBM.

TMZ primarily exerts its cytotoxicity by forming O^6-alkylguanine DNA adducts. MGMT, the DNA repair enzyme, plays a crucial role in removing these DNA lesions at O^6-guanine, contributing significantly to resistance against alkylating agents. We noticed a link between HOTAIR-mediated TMZ resistance and increased MGMT protein levels mediated by the HOTAIR/miR-214-3p/β-catenin network. This observation suggests that HOTAIR serves as a ceRNA, increasing β-catenin transcription by sequestering and thus inhibiting miR-214-3p activity. This reasoning concurs with previous research reporting activation of the HOTAIR/miR-214-3p/β-catenin network in cervical cancer [29]. The regulatory activity of HOTAIR on β-catenin expression has also been found in U87 GBM cell line, in which HOTAIR knockdown also inhibited β-catenin expression [34]. Beta-catenin serves as a transcriptional co-activator that interacts with transcription factors of the lymphoid enhancer factor/T cell factor (LEF/TCF) family. This interaction induces the transcription of target genes and is a downstream mediator of the Wnt signaling pathway [35, 36]. Dysregulation of the Wnt/β-catenin pathway is commonly noticed in many types of tumors and is strongly linked to

tumorigenesis, metastatic progression, and chemoresistance [37, 38]. There are several putative LEF/TCF binding sites at the 5′-upstream region of the human MGMT gene. The regulatory activity of β-catenin on MGMT transcription has also been reported in colon carcinoma cells [39]. Our study indicates that β-catenin signaling plays a crucial role in HOTAIR-induced TMZ resistance, with MGMT acting as a downstream molecule that directly affects the response to TMZ treatment.

We also noticed a significant correlation between HOTAIR expression and *MGMT* promoter methylation. DNA methylation is a key mechanism in epigenetic silencing. Patients with a methylated *MGMT* promoter exhibit lower HOTAIR expression than those with an unmethylated promoter. This finding suggests a possible effect of HOTAIR on the methylation status of *MGMT*. Thus, in addition to its β-catenin-dependent effect, HOTAIR may also contribute to increased *MGMT* transcription through DNA methylation. However, further studies are needed to confirm whether HOTAIR promotes the unmethylated status of the *MGMT* promoter. Methylation of the *MGMT* promoter is a well-established predictor of GBM patient survival and their response to TMZ chemotherapy [40]. However, the routine implementation of this biomarker in clinical practice remains

a challenge owing to differences in detection methods used across laboratories, resulting in no consensus on the best method or standardized cut-off definitions for *MGMT* methylation status [41]. Moreover, MGMT protein levels do not consistently align with methylation status because their expression is regulated by multiple mechanisms beyond promoter methylation. Thus, the combination of *MGMT* methylation and HOTAIR expression may provide a more reliable predictor of TMZ sensitivity. Furthermore, several observations suggest that HOTAIR has the potential to serve as a serum biomarker for several tumors, including GBM [42-45]. Thus, monitoring serum HOTAIR levels dynamically during treatment could serve as peripheral biomarkers for predicting TMZ response and tumor recurrence. However, further studies are required to establish a standardized testing method and cutoff definition for high HOTAIR expression.

We identified MTX as a potential therapeutic agent for patients with GBM with high HOTAIR expression. MTX is a potent competitive antagonist of dihydrofolate reductase and was first available in the 1950s. It suppresses the formation of tetrahydrofolate, subsequently inhibiting the synthesis of purines and thymidylate; this disruption leads to impaired cell replication

and the blockade of cell cycle progression from the G1 to S phase. MTX is widely used as a chemotherapeutic agent for leukemia, lymphoma, and breast cancer [46]. Although MTX has limited ability to penetrate the blood-brain barrier, several strategies have been applied to achieve an adequate dose within the CNS. These include systemic administration at a high dose, intra-arterial delivery following pharmacological disruption of the blood-brain barrier, and intrathecal administration [47]. High-dose MTX-based polychemotherapy has shown benefits for patients with primary CNS lymphoma and brain metastases [31, 32]. In a phase II trial involving pediatric glioma, two cycles of high-dose MTX pre-treatment prior to chemoradiotherapy improved event-free survival and the response to chemoradiotherapy when compared to MTX-free protocols [48]. High-dose MTX treatment is well-tolerated with leucovorin (folinic acid) rescue therapy and other preventive measures, such as vigorous hydration and urine alkalinization before MTX infusion. Therefore, MTX chemotherapy is a feasible and safe treatment option for CNS disorders. In the present study, we found that MTX treatment increased TMZ sensitivity and decreased HOTAIR expression in U251R cells. This observation is consistent with reports of HOTAIR downregulation following MTX treatment in

patients with rheumatoid arthritis [49]. Our results indicate that MTX holds promise as a chemotherapy option for patients with GBM with high HOTAIR expression and that a combination of MTX and TMZ may benefit TMZ-resistant patients with GBM.

This study has certain limitations. First, the mechanism underlying HOTAIR upregulation in TMZ-resistant GBM cells was not assessed. Further, previous research indicates that HOTAIR is upregulated in gliomas via epigenetic (DNA hypermethylation) and transcriptional mechanisms (transcription factors, including bromodomain containing 4, HOXA9, and c-Myc) [50]. However, whether similar mechanisms come into play during the development of TMZ resistance warrants further investigation. Second, we noticed that gliomas with mutant IDH1 or 1p19q co-deletions had lower HOTAIR expression than those with wild-type IDH1 or non-co-deleted 1p19q. Notably, gliomas with these mutations usually have more favorable outcomes. However, it remains unclear whether these mutations affect HOTAIR expression.

In conclusion, our study highlights that elevated HOTAIR expression is a risk factor for TMZ resistance in glioblastoma, primarily by activating the miR-214/β-catenin/MGMT pathway. This study also advocates for the repurposing of MTX as an effective drug

for glioma treatment, especially in cases with high HOTAIR expression status. However, before the latter is achieved, future studies are needed to assess MTX efficacy in the treatment of GBM in vivo.

Materials and methods

Reagents

TMZ and methotrexate (MTX) were purchased from MedChemExpress (Monmouth Junction, NJ, USA), and SKL2001 was purchased from Selleck (Houston, TX, USA). These chemicals were dissolved in DMSO and stored at -20°C. The dose of SKL2001 (20 μM) used in this study was chosen according to a previou study[23]. Lipofectamine 3000 was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Primary antibodies against β-catenin and O^6-methylguanine-DNA methyltransferase (MGMT) were purchased from Abcam (Cambridge, UK), and antibodies against GAPDH and H3 were obtained from Cell Signaling Technology (Danvers, MA, USA) and Bioss (Beijing, China), respectively.

Bioinformatic analysis of TMZ resistance-associated lncRNAs from public datasets

We collected data on lncRNA expression in TMZ-resistant U251 (GSE100736) and LN299 (GSE113510) cell lines in addition to three

pairs of human primary and recurrent GBM specimens from the GSE7696 dataset. All data were downloaded from http://www.ncbi.nlm.nih.gov/geo. Patients with GBM from the GSE7697 dataset experienced recurrence after undergoing surgical resection with TMZ and radiotherapy. The differentially expressed lncRNAs between TMZ-resistent and TMZ-sensitive cell lines or primary and paired recurrent GBM were identified using the “Limma” R package with the cut-off criteria of fold change $|(\log_2 FC)| > 1$ and an adjusted $P < 0.05$, and visualized using the “pheatmap” R package.

Bioinformatic analysis of the association between HOTAIR expression and clinical characteristics from public databases

We collected data on HOTAIR expression and clinical information from The Cancer Genome Atlas (TCGA; http://xena.ucsc.edu), Chinese Glioma Genome Atlas (CGGA; http://www.cgga.org.cn), and GSE16011 (http://gliovis.bioinfo.cnio.es). These patients were reclassified according to the fifth edition of the WHO classification of tumors of the central nervous system[17]. Then patients were classified into high and low subgroups based on the median expression level of HOTAIR. The correlation between HOTAIR expression and clinicopathological features was measured using the permutation test. Survival analysis was plotted using Kaplan–Meier

curve followed by the log-rank test. The HOTAIR-related biological pathways were estimated by gene set variation analysis (GSVA) [51], and the enriched gene sets were defined when the adjusted *P* value was less than 0.05, along with an enrichment score of >6 in the TCGA database or >3 in the CGGA database.

Estimation of drug response and prediction of potential therapeutic agents

First, each tumor sample expression profile from TCGA was purified using ISOpure to estimate the fraction of tumor cells and provide a purified expression profile of the tumor cells [52]. Subsequently, we estimated the drug response for each sample based on two pharmacogenomic datasets, the Cancer Therapeutics Response Portal (CTRP, https://portals.broadinstitute.org/ctrp) and the PRISM Repurposing dataset (http://depmap.org/portal/prism/), which contain sensitivity data for 481 compounds in 853 human cancer cell lines and 1448 compounds in 482 cancer cell lines, respectively. Drug sensitivity was measured using the area under the curve (AUC) values of the dose-response curve, where lower AUC values indicated higher sensitivity to the compounds.

The drug response of each sample from TCGA was estimated using the ridge regression model calculated with the “pRRophetic” R

package [53]. The compounds with higher sensitivity (log_2FC >0.12 for CTRP and log_2FC >0.08 for PRISM) in high HOTAIR-expressing patients were identified by comparing the drug response between high- (top decile) and low-HOTAIR (bottom decile) groups. Meanwhile, Spearman's correlation analysis between AUC values and HOTAIR expression was performed to select the compounds with negative correlation coefficients (Spearmen's r < -0.28 for CTRP; Spearmen's r <-0.3 for PRISM).

Cell culture and establishment U251R cells

Human U251 GBM cells were obtained from the China Center for Type Culture Collection (Wuhan, China). The cells were cultured with high-glucose Dulbecco's modified Eagle's medium (Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (Gibco). U251R cells were established by exposing U251 cells to incremental concentrations of TMZ (100, 200, 300, and 400 μM) for 4 months. The cells were cultured with each concentration of TMZ for 3 wk and the final surviving cells were cultured in a medium containing 400 μM TMZ for an additional month, replacing medium every two days. Afterward, U251R cells were maintained in a medium containing 200 μM TMZ.

Cell transfection

Full-length cDNA of human HOTAIR were synthesized and cloned into the pcDNA3.1 plasmid (Sangon Biotech, Shanghai, China). The small interference RNA (siRNA) targeting HOTAIR (5′-GTACCGACCTGGTAGAAAA-3′) and the scrambled control were purchased from RiboBio (Guangzhou, China). The miR-214-3p mimic (5′-ACAGCAGGCACAGACAGGCAGU-3′) and the negative control were purchased from General Biology (Anhui, China). Cells were transfected with Lipofectamine 3000 and used for subsequent experiments after 48 h of transfection.

Cell viability assay

The cells were seeded in 96-well plates at a density of 3×10^3 cells/well in 100 μL of medium and grown for 24 h. After 48 h of chemicals treatment, 10 μL CCK-8 solution (Vazyme, Nanjing, China) was added and incubated at 37°C for 1 h. The absorbance at 450 nm was measured using a spectrophotometer (BioTek, Winooski, VT, USA).

Quantitative RT-PCR

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and reverse transcribed using a cDNA synthesis kit (Toyobo, Osaka, Japan) or miScript RT kit (Vazyme, Nanjing, China). Quantitative RT-PCR was performed using SYBR Green Supermix

(Vazyme, Nanjing, China) according to the manufacturer's instructions. The relative expression of the target genes was calculated using $2^{-\Delta\Delta CT}$ method, and GAPDH or U6 were used as internal references. The primers used are listed in Supplementary file 1.

Western blotting

Total protein and cytoplasmic and nuclear protein fractions were extracted using RIPA buffer and nuclear and cytoplasmic protein extraction kits (Beyotime Biotechnology, Shanghai, China), respectively. The protein concentration was measured using bicinchoninic acid protein assay kit (Merck Millipore, MA, USA). Proteins were separated on 10% acrylamide gels and transferred onto polyvinylidene fluoride membranes (Merck Millipore, MA, USA). After blocking with 5% bovine serum albumin for 30 min at room temperature, the membranes were incubated with primary antibodies overnight at 4°C and subsequently, with secondary antibodies for 2 h at room temperature. Finally, the blots were visualized using an enhanced chemiluminescence reagent (Vazyme). The density of the bands was analyzed using ImageJ software and expressed relative to GAPDH or H3.

Flow cytometry analysis

The Cell Cycle Staining Kit (Multisciences, Hangzhou, China) and Annexin V-FITC/PI kit (Vazyme) were used for the analysis of cell cycle and apoptosis, respectively. Flow cytometry was performed using CytoFLEX (Beckman Coulter, Brea, CA, USA) and analyzed using FlowJo software.

Statistical analysis

R statistical package (version 4.0.2) was used for bioinformatic c analysis. In the section on cell culture, all data presented are mean ± SD, and the statistical analysis was conducted using GraphPad Prism 8.4.0 (GraphPad Software Inc., San Diego, CA, USA). Group comparisons were evaluated using the Student's *t*-test or one-way analysis of variance (ANOVA), followed by Tukey's post-hoc test for multiple comparisons. Comparisons of cell viability under TMZ or MTX treatment with increasing concentrations were evaluated using two-way ANOVA. Additionally, the half-maximal inhibitory concentration (IC50) values were determined by nonlinear regression analysis of the dose-response curve. Statistical significance was set at P-value < 0.05.

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Supplementary files

Supplementary file 1: The primer sequences for QPCR

Supplementary file 2: Original data for Western Blot

List of abbreviations

HOTAIR: HOX transcript antisense RNA; GBM: glioblastoma; TMZ: temozolomide; MGMT: O^6-methylguanine-DNA methyltransferase; IDH1: isocitrate dehydrogenase 1; MTX: methotrexate

Consent for pulication

Not applicable

Availability of data and materials

All data that support the findings of this study are available in the manuscript. The datasets analysed during the current study are publicly available and the accession numbers for the datasets are presented in the manuscript.

Competing interests

The authors declare that they have no competing interests.

Ethics declarations

Not applicable

Fundings

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Authors’ contribution

TL performed experiments and prepared the first draft of the manuscript. DHY helped with bioinformatics analysis. WQ and XC helped with part of experiments and figures preparation. ZFW and ZQL designed and supervised the study and revised the manuscript. All authors have seen and approved the final version of the manuscript being submitted.

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Figure legends

Figure 1. LncRNA HOTAIR is highly expressed in TMZ-resistant GBM cells and recurrent GBM tissues. A: Upregulated lncRNAs in GSE100736, GSE113510, and GSE7696. B: Intersection of the upregulated lncRNAs in the three datasets. C: Expression of HOTAIR in glioma with different grades in TCGA, CGGA, and GSE16011 cohorts. D: Overview of the association between HOTAIR expression and clinicopathologic characteristics in TCGA, CGGA, and GSE16011 cohorts. E: Patients with glioma with high HOTAIR expression had a shorter overall survival than those with low HOTAIR expression. F: Survival analysis in patients receiving TMZ chemotherapy showed that patients with high HOTAIR expression

exhibited a shorter overall survival than those with low expression levels.

Figure 2. HOTAIR silencing restored TMZ sensitivity in U251R cells and HOTAIR overexpression conferred TMZ resistance in U251 cells. A: The established U251R cells had a higher IC_{50} of TMZ than parental U251 cells (n=4). The cells were treated with TMZ for 48 h, and then cell viability was measured using CCK8 assay. IC50 was caculated by nonlinear regression analysis of the dose-response curve. B: HOTAIR expression is upregulated in U251R cells compared with that in U251 cells (n=5). C: HOTAIR expression was downregulated in U251R cells transfected with si-HOTAIR (n=6). D: Silencing HOTAIR increased the sensitivity of U251R cells to TMZ treatment (D, n=4). E-G: Silencing HOTAIR increased TMZ(400 μM, 48 h)-induced G2/M arrest (E, n=4) and apoptosis (F and G, n=5) in U251R cells. H-K: HOTAIR overexpression (H, n=6) decreased TMZ sensitivity (I, n=4) and reduced TMZ(200 μM, 48 h)-induced G2/M arrest (J, n=4) and apoptosis (K, n=6) in U251 cells. The doses of TMZ that used to induce apoptosis were chosen because they had about 30% cell viability inhibition in U251 and U251R cells.

Figure 3. HOTAIR regulated MGMT expression. A, B: Silencing HOTAIR reduced MGMT mRNA (A, n=6) and protein (B, n=5) levels

in U251R cells. C, D: HOTAIR overexpression increased MGMT mRNA (C, n=6) and protein (D, n=6) levels in U251 cells.

Figure 4. HOTAIR-mediated TMZ resistance was associated with the β-catenin/MGMT pathway. A: An overview of GSVA scores for pathway activities between high and low HOTAIR expression groups in TCGA and CGGA cohorts. B: U251R cells had higher total β-catenin protein content than U251 cells (n=4). C: Silencing HOTAIR reduced the total (n=6), cytosolic (n=5), and nuclear (n=5) β-catenin protein content in U251R cells. D: HOTAIR overexpression increased the total (n=6), cytosolic (n=5), and nuclear (n=5) β-catenin protein content in U251 cells. E, F: SKL2001 (20 μM, 12h) treatment increased β-catenin protein level in the whole cell lysate and cytosolic and nuclear fractions (E, n=4) as well as MGMT protein level (F, n=6) in HOTAIR silencing-U251R cells. G, H: Pre-treatment with SKL2001 (20μM, 12h) reduced TMZ (400 μM, 48 h)-induced G2/M arrest (G, n=3) and apoptosis (H, n=5) in HOTAIR- silencing U251R cells. The cells were pre-treated with SKL2001 for 12 h, followed by 48 h of TMZ treatment.

Figure 5. HOTAIR activated β-catenin/MGMT pathway via sponging miR-214. A: U251R cells had a lower miR-214 level than U251 cells (n=5). B: Silencing HOTAIR in U251R cells increased

miR-214 level and overexpressing HOTAIR in U251 cells decreased miR-214 level, indicating an inversely correlation between HOTAIR expression and miR-214 level (n=6). C, D: The miR-214-3p mimics increased TMZ-induced G2/M arrest and apoptosis in U251R cells (n=3). E: The miR-214-3p mimics reduced β-catenin and MGMT protein levels in U251R cells (n=5).

Figure 6. MTX increased TMZ sensitivity and reduced the expression of HOTAIR and β-catenin in U251R cells. A: The prediction of potential therapeutic compounds for patients with high HOTAIR expression based on the drug sensitivity data from CTRP (upside panel) and PRISM (downside panel). The lower AUC values indicate higher sensitivity to the compounds. ***P<0.001. B: MTX treatment significantly reduced the viability of U251R cells (n=5). The dose of 0.4 μM that showed an approximate 50% cell viability inhibition was used for subsequent expeiments. C: MTX pre-treatment (0.4 μM, 12 h) increased the sensitivity of U251R cells to TMZ (n=5). U251R cells were pre-treated with 0.4 μM MTX for 12 h, and then cells were washed with PBS and cultured in media containing TMZ for 48 h. D, E: MTX (0.4 μM, 48 h) caused G1 phase arrest (D, n=3) and cell apoptosis (E, n=4) in U251R cells. F, G: MTX

(0.4 μM) treatment reduced HOTAIR mRNA and β-catenin protein levels in U251R cells (n=5).

Figures

Figure 2

Supplementary Files

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- Supplementaryfile1.docx
- Supplymentfile2.pptx.zip