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Gene Expression Profiling Regulated by lncRNA H19 Using Bioinformatic Analyses in Glioma Cell Lines

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Abstract. *Background/Aim: Glioma, the most common type of primary brain tumor, is characterized by high malignancy, recurrence, and mortality. Long non-coding RNA (lncRNA) H19 is a potential biomarker for glioma diagnosis and treatment due to its overexpression in human glioma tissues and its involvement in cell division and metastasis regulation. This study aimed to identify potential therapeutic targets involved in glioma development by analyzing gene expression profiles regulated by H19. Materials and Methods: To elucidate the role of H19 in A172 and U87MG glioma cell lines, cell counting, colony formation, and wound healing assays were conducted. RNA-seq data analysis and bioinformatics analyses were performed to reveal the molecular interactions of H19. Results: Cell-based experiments showed that elevated H19 levels were related to cancer cell survival, proliferation, and migration. Bioinformatics analyses identified 2,084 differentially expressed genes (DEGs) influenced by H19 which are involved in cancer progression. Specifically, ANXA5, CLEC18B, RAB42, CXCL8, OASL, USP18, and CDCP1 were positively correlated with H19 expression, while CSDC2 and FOXO4 were negatively correlated. These DEGs were predicted to function as oncogenes or tumor suppressors in gliomas, in association with H19. Conclusion: These findings highlight H19 and its associated genes as potential diagnostic and therapeutic targets for gliomas, emphasizing their clinical significance in patient survival.*

Among adult brain tumors, gliomas are the most common primary malignancy, constituting approximately 80% of all cases (1). Gliomas include all tumors derived from neuroglial progenitor cells (2) and are categorized into two types based on histological features, low-grade gliomas (LGGs) and high-grade gliomas (HGGs). The most aggressive form of HGG is glioblastoma multiforme (GBM), a particularly malignant tumor with a median survival time of 12 months (3). Due to their high malignancy, recurrence, and mortality rates are the most representative features of gliomas, a variety of new cancer treatments, such as surgery, chemotherapy, and radiation therapy, have been proposed and combined to improve outcomes (4-6). However, despite treatment advancements, the treatment success rate for glioma remains low, and some treatments are associated with serious side effects (7, 8). Studies on the pathogeneses of gliomas have identified molecular mechanisms associated with brain tumors and support the potential of personalized cancer therapy (9, 10). Therefore, to facilitate the discovery of effective biomarkers, it is essential to profile and analyze gene expression changes in glioma patients.

Long non-coding RNAs (lncRNAs) are non-coding RNAs that are greater than 200 nucleotides in length and do not translate into proteins (11, 12). LncRNAs have specific biological roles in the nucleus and cytoplasm (13, 14) and are

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Table I. *Function of H19 gene/protein in various tumor types.*

Cancer type	Cancer correlation	Effects on cancer	Ref.
Breast cancer	Positive	Increasing the aggressiveness of breast cancer and promoting migration, tumor growth, and metastasis	(24)
Gastric cancer	Positive	Inducing cell proliferation and invasion, and inhibiting cell apoptosis through sponging for miR-138 and up-regulation of E2F2	(26)
Glioma	Positive	Reinforcing cell proliferation, invasion, and migration *via* Wnt/β-catenin signaling pathway	(27)
Lung adenocarcinoma	Positive	Promoting cell proliferation and EMT, and inhibiting apoptosis through increased methylation of CDH1 promoter	(28)
Liver tumor	Negative	Inhibiting tumor growth and development in liver	(29)
Osteosarcoma	Negative	Accelerating DNA repair process through down-regulation of SNORA7A	(30)
Papillary thyroid carcinoma	Negative	Reducing cell proliferation and migration through down-regulation of TNFR2	(31)

CDH1: Cadherin 1; EMT: epithelial mesenchymal transition; E2F2: E2F transcription factor 2; SNORA7A: small nucleolar RNA, H/ACA box 7A; TNFR2: tumor necrosis factor receptor 2.

concerned with chromatin and histone modification, mRNA splicing, and microRNA (miRNA) processing, and by so doing, regulate nuclear gene expressions. However, they have also been reported to act as competitive endogenous RNAs (ceRNAs) in the cytoplasm, to function as miRNA sponges, and to regulate mRNA expressions (13, 15, 16). In addition, lncRNAs contribute significantly to cancer development by regulating major signaling pathways, such as NF-κB, p53, Notch, and PI3K/AKT (17, 18). Dysregulation of lncRNAs is closely associated with tumor progression, metastasis, and metabolic reprogramming (19-21).

H19 is one of the most widely studied lncRNAs, with its gene located on chromosome 11p15.5 (22, 23). Previous studies have shown that H19 levels vary and exhibit tumor-promoting or preventing activity depending on cancer type (Table I). Elevated H19 expression in breast cancer significantly correlates with enhanced tumorigenesis, accelerated progression, and resistance to chemotherapies, including temozolomide therapy (24, 25). H19 up-regulation is positively correlated with cell invasion and tumor growth in gastric cancer (26), while it activates signaling pathways associated with cell proliferation and migration in glioma (27), and promotes epithelial-mesenchymal transition (EMT) in lung adenocarcinoma (28). On the other hand, other studies have reported that H19 has a tumor-suppressive role. For example, lack of H19 expression has been associated with liver carcinogenesis (29). In osteosarcoma, H19 expression has been negatively correlated with tumorigenesis and promoted DNA repair by inhibiting the activity of the small nucleolar RNA H/ACA box 7A (SNORA7A) (30). Additionally, H19 acts as a negative regulator of tumor necrosis factor receptor 2 (TNFR2) expression in papillary thyroid carcinoma, thereby suppressing cell proliferation and migration (31). Taken together, H19 has various effects in cancers; nevertheless, in glioma, it is known to be overexpressed and associated with angiogenesis, proliferation, metastasis, and EMT by interacting with several miRNAs. Thus, H19 is a great candidate to be investigated as a potential therapeutic target for glioma progression, diagnosis and facilitating the development of novel therapeutic strategies (22, 32).

Recently, bioinformatics has been utilized to screen for cancer-related genes, including H19, and integrated analyses of multi-omics data have identified cancer-specific biomarkers and potential therapeutic targets for anti-cancer drugs (25, 33, 34). These bioinformatics tools enable the exploration of genes and signaling pathways and the analysis of clinical data, such as relationships between gene expression levels and their effects on individual cancer patients. Bioinformatics analyses are effective tools for gaining a deeper understanding of functional molecules and biological networks in different cancer types, and the results of these analyses can be used to create cancer-specific gene lists and provide valuable predictions for clinical studies.

In this study, transcriptome sequencing analysis was conducted on glioma cell lines transfected to overexpress or knockdown H19 to identify differentially expressed genes (DEGs) that are significantly associated with H19 expression and simultaneously closely related to tumorigenesis. Furthermore, bioinformatics analyses of these DEGs were used to validate relationships between the altered expressions of selected genes and the characteristics of glioma. This study focused on investigating the impact of H19 in gliomas and to identify molecular mechanisms of therapeutic and diagnostic utility.

Materials and Methods

Cell culture and transient transfection. The A172 and U87MG human glioma cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). A172 cells were cultured in RPMI-1640 (Hyclone, Marlborough, MA, USA) containing 10% FBS (Hyclone), 100 U/ml penicillin, and 100 μg/ml

streptomycin (Gibco, Waltham, MA, USA) in a humidified incubator with a 5% CO_2/95% air atmosphere at 37˚C, whereas U87MG cells were incubated in MEM (Hyclone) under the same conditions (35).

The two cell lines were set into four groups: negative control (NC), siRNA control (si-Cont), H19-knockdown (H19-KD), and H19-overexpression (H19-OE) groups. Transient transfection was performed when cell confluence reached 70-80%. The si-Cont group was transfected with scrambled siRNA (Invitrogen, Waltham, MA, USA), the H19-KD group with H19-specific siRNA (Thermo Scientific, Waltham, MA, USA) using Lipofectamine RNAiMAX (Invitrogen), and the H19-OE group with pcDNA3.1-H19 vector using Lipofectamine 2000 (Invitrogen). Cells were harvested after transfection for 48 h for cell-based assays.

Total RNA-preparation. After transiently transfecting each glioma cell line with H19-specific siRNA or pcDNA3.1-H19 vector, total RNA was extracted using TRI reagent (Invitrogen), as previously described (36). RNA qualities and concentrations were assessed using OD 260/280 ratios using a Nanodrop 2000 (Thermo Scientific). Extracted RNAs were subjected to reverse transcription quantitative polymerase chain reaction (RT-qPCR) and forwarded to Macrogen Inc. (Seoul, Republic of Korea) for transcriptome sequencing.

RT-qPCR. The M-MLV reverse transcriptase system (Bioneer, Daejeon, Republic of Korea) was used to generate first-strand cDNAs from total RNA. The conditions used for RT were 60 min at 37˚C and 5 min at 99˚C and were stored at 4˚C for subsequent experiments. RT products were immediately subjected to qPCR. Real-time qPCR of the RT products was performed using GoTaq® qPCR Master Mix (Promega, Madison, WI, USA) according to the manufacturer's instructions and normalized *versus* U6 (37). The qPCR protocol used in this study included the following steps: initial activation of GoTaq Hot Start Polymerase at 95˚C for 2 min, followed by 40 cycles of denaturation at 95˚C for 15 sec, and annealing and extension at 60˚C for 1 min. The relative expression levels of RNAs were calculated using the comparative $2^{-\Delta\Delta Ct}$ method (38). The primers used for this study were as follows: H19 forward primer: 5'-TTCAAAGCCTCCACGACTCT-3'; H19 reverse primer: 5'-CTGAGACTCAAGGCCGTCTC-3'; U6 forward primer: 5'-CTCGCTTCGGCAGCACATA-3'; U6 reverse primer: 5'-CGAATTTGCGTGTCATCCT-3'.

Cell counting assay. Trypan blue solution (Gibco) was used to determine numbers of living cells. In brief, cells (1×10^5) were incubated in 60 mm dishes for 24 h, transfected with si-H19 or pcDNA3.1-H19 vector, washed twice with PBS (WELGENE, Gyeongsan, Gyeongbuk, Republic of Korea), detached using 10% trypsin-EDTA (Gibco), resuspended in PBS, and diluted 1:1 with trypan blue solution (39). Numbers of surviving cells were then measured to assess short-term cell viability.

Colony formation assay. A172 and U87MG cells were seeded at 3,000 cells per 35 mm dish, incubated for 48 h, transfected with si-H19 or pcDNA3.1-H19 vector, and incubated in the same conditions as cell culture in a humidified incubator for 7 days. Resulting colonies were washed twice with PBS (WELGENE), fixed with 50% methanol, and stained with 0.5% crystal violet (Sigma, St. Louis, MO, USA) (40). Colonies containing more than 50 cells were then imaged and scored as survivors to assess long-term cell viability.

Wound healing assay. Wound healing assays were conducted to analyze the effects of H19 on cell migration (41). Transfection was performed after cells had achieved 80% confluence in 60 mm dishes at 37˚C in a 5% CO_2 atmosphere. Cell layers were scratched with a sterile yellow tip, washed twice with PBS to remove floating cells and incubated in fresh culture medium for 48 h. Photomicrographs were captured at ×100 using an Olympus CKX53 inverted microscope (Olympus Optical, Tokyo, Japan).

Transcriptome analysis. Total RNA concentrations were calculated using the Quant-IT RiboGreen assay (Invitrogen). To evaluate the integrity of total RNA, samples were analyzed using the TapeStation RNA screentape (Agilent, Santa Clara, CA, USA). Only high-quality RNA preparations, with RIN values greater than 7.0 were utilized to construct RNA libraries. For each sample, an independent library was prepared using 0.5 μg of total RNA and the Illumina TruSeq Stranded Total RNA Library Prep Gold Kit (Illumina, Inc., San Diego, CA, USA). rRNA was removed from total RNA using the Ribo-Zero rRNA Removal Kit (Human/Mouse/Rat Gold) (Illumina), and the remaining mRNA was fragmented under elevated temperature conditions using divalent cations. Cleaved RNA fragments were then converted into first-strand cDNA using SuperScript II reverse transcriptase (Invitrogen) and random primers. Subsequently, second-strand cDNA synthesis was performed using DNA Polymerase I, RNase H, and dUTP. cDNA fragments were end-repaired, and this was followed by the addition of a single 'A' base and adapter ligation. The resulting products were purified, and PCR was used to enrich the final cDNA libraries. Libraries were quantified using KAPA Library Quantification Kits for Illumina Sequencing platforms as detailed in the qPCR Quantification Protocol Guide (Kapa Biosystems Inc., Wilmington, MA, USA). Library quality was assessed using the TapeStation D1000 ScreenTape (Agilent). Indexed libraries were then submitted for sequencing on an Illumina NovaSeq (Illumina), and paired-end (2×100 bp) sequencing was performed by Macrogen Inc. The sequencing results were deposited in the Gene Expression Omnibus database (GEO Series accession number GSE243116).

RNA-seq data analysis. The raw reads obtained from the sequencer underwent preprocessing to eliminate low-quality and adapter sequences prior to analysis. Processed reads were aligned to the *Homo sapiens* (hg38) reference genome using HISAT v2.1.0 (42), which utilizes two types of indexes for alignment, a global, whole-genome index and tens of thousands of small local indexes. These indexes are constructed using the Burrows-Wheeler transform and a graph FM index similar to Bowtie2. The efficient data structures and algorithms of HISAT enable faster spliced alignments compared to widely used tools like Bowtie and Burrows-Wheeler Aligner. The reference genome sequence of *Homo sapiens* (hg38) and annotation data were obtained from the UCSC table browser (http://genome.uscs.edu). Following alignment, StringTie v2.1.3b (43, 44) was utilized to assemble the aligned reads into transcripts and estimate transcript abundances. The resulting data provided relative abundance estimates as Read Count values for each transcript and gene expressed in each sample.

Statistical analysis was performed to identify DEGs based on the abundance estimates for each gene across samples. Genes with zero or fewer Read Count values in all samples were excluded. To facilitate $\log_2$ transformation, a value of 1 was added to each Read Count value for filtered genes. Filtered data was then $\log_2$-transformed and subjected to relative log expression normalization.

The statistical significances of differential expression data were assessed using the nbinomWaldTest function in DESeq2, considering fold change. The null hypothesis assumed no intergroup difference. *p*-Values were adjusted using the Benjamini-Hochberg algorithm to control the false discovery rate. Hierarchical clustering analysis was performed on the set of DEGs using complete linkage and Euclidean distance as a measure of similarity.

Bioinformatic analyses of DEGs. Gene ontology (GO) enrichment analyses (45, 46) and Kyoto encyclopedia of genes and genomes (KEGG) pathway analyses (47-49) were conducted on significant gene lists utilizing DAVID 2021 (https://david.ncifcrf.gov). These analytical approaches offer comprehensive functional genomic annotations (50, 51). Gene expression and survival analysis were subsequently performed to validate expression levels in glioma *versus* normal tissue using GEPIA2 (http://gepia2.cancer-pku.cn/) leveraging data from the cancer genome atlas (TCGA) and the genotype-tissue expression (GTEx) databases (52).

Statistical analysis. Statistical analysis was conducted using Prism 5 software (GraphPad Software, San Diego, CA, USA), and *p*-values of <0.05 were considered significant. Each experiment was performed independently in three technical replicates per sample, and results were analyzed by the two-tailed unpaired Student's *t*-test, one-way ANOVA for ranked data followed by Tukey's honestly significant difference test, or two-way ANOVA for ranked data followed by Bonferroni's post-tests.

Results

Effects of H19 on glioma cell lines. In order to study the effects of H19 in glioma, cell survival, proliferation, and migration assays were performed on A172 and U87MG glioma cell lines. First, RT-qPCR was used to assess H19 expression levels by comparing H19-OE groups and H19-KD groups with NC groups for each cell line. The results demonstrated a significant reduction in H19 expression in the H19-KD groups compared to the NC groups, whereas H19 expression was notably elevated in the H19-OE groups (Figure 1A). Cell counting assays showed cell viability was significantly greater in the H19-OE groups but significantly lower in the H19-KD groups (Figure 1B), and colony formation assays showed colony numbers were significantly higher in the H19-OE groups but significantly lower in the H19-KD groups (Figure 1C). Wound healing assays revealed percentages of covered areas were higher in the H19-OE groups but lower in the H19-KD groups (Figure 1D). Taken together, these findings indicate that H19 up-regulation in glioma promotes tumorigenesis, while its down-regulation suppresses tumorigenesis, and also confirm the role of H19 as an oncogenic factor in glioma.

Profiling of H19-regulating DEGs in glioma cell lines. Glioma cell lines (A172, U87MG) were treated with si-H19 or pcDNA3.1-H19 vector, respectively, to identify changes in gene expression regulated by H19. Transient transfection was performed for each cell line, resulting in H19-KD groups and H19-OE groups for A172 and two corresponding groups for U87MG. Comparative analysis was performed based on total RNA sequencing analysis data to determine the fold changes shown by DEGs. Analysis showed that 242 DEGs in A172 and U87MG cell lines were down-regulated in the H19-KD groups, and 319 DEGs were up-regulated ($|\log_2$ fold change$| \geq 1.5$) (Figure 2A). In contrast, 875 DEGs were up-regulated in the H19-OE groups, and 648 DEGs were down-regulated ($|\log_2$ fold change$| \geq 1.5$) (Figure 2B). The results showed that a comprehensive set of 2,084 DEGs, including both protein-coding RNAs and non-coding RNAs, were regulated by H19. 1,379 protein-coding DEGs from the total were selected for functional analysis. Among these genes, 74 DEGs down-regulated in the H19-KD groups and 539 DEGs up-regulated in the H19-OE groups were expected to be oncogenic (Figure 2C) and 118 DEGs up-regulated in the H19-KD groups and 648 DEGs down-regulated in the H19-OE groups were expected to be tumor suppressive (Figure 2D), based on the tumorigenic roles of H19 (8, 53).

Functional analyses of DEGs regulated by H19. Functional analyses, including GO and KEGG pathway enrichment analyses, were conducted on 613 protein-coding DEGs predicted to have oncogenic properties. GO enrichment analyses unveiled notable enrichment of these DEGs within categories such as 'DNA binding' (112 genes, p=1.35E-05) under Molecular Function, 'Chromosome' (111 genes, p=1.93E-09) under Cellular Component, and 'Cell cycle' (111 genes, p=2.12E-13) under Biological Process (Figure 3A). KEGG pathway analyses demonstrated high levels of association between the selected DEGs and pathways such as 'Pathways in cancer' (30 genes, p=4.0E-03), 'PI3K-Akt signaling pathway' (19 genes, p=3.9E-02), 'Ras signaling pathway' (15 genes, p=2.2E-02), and 'Viral carcinogenesis' (14 genes, p=3.4E-02) (Figure 3B). In addition, GO and KEGG pathway enrichment analyses were performed on 766 protein-coding DEGs predicted to be tumor suppressors. The GO enrichment analyses demonstrated that selected DEGs exhibited predominant enrichment in 'Ion binding' (293 genes, p=6.65E-06) under Molecular Function, 'Neuron part' (94 genes, p=7.79E-04) under Cellular Component, and 'Nervous system development' (131 genes, p=1.86E-05) under Biological Process (Figure 3C). Besides, the KEGG pathway enrichment analyses showed close associations between the selected DEGs and several key pathways such as 'cAMP signaling pathway' (17 genes, p=6.8E-03), 'FOXO signaling pathway' (12 genes, p=7.2E-03), 'AMPK signaling pathway' (12 genes, p=4.0E-03), and 'Longevity regulating pathway' (11 genes, p=1.2E-03) (Figure 3D).

Bioinformatic analyses of DEGs interacting with H19 in gliomas. A total of 1,379 candidate DEGs, associated with glioma and expected to interact with H19, were screened.

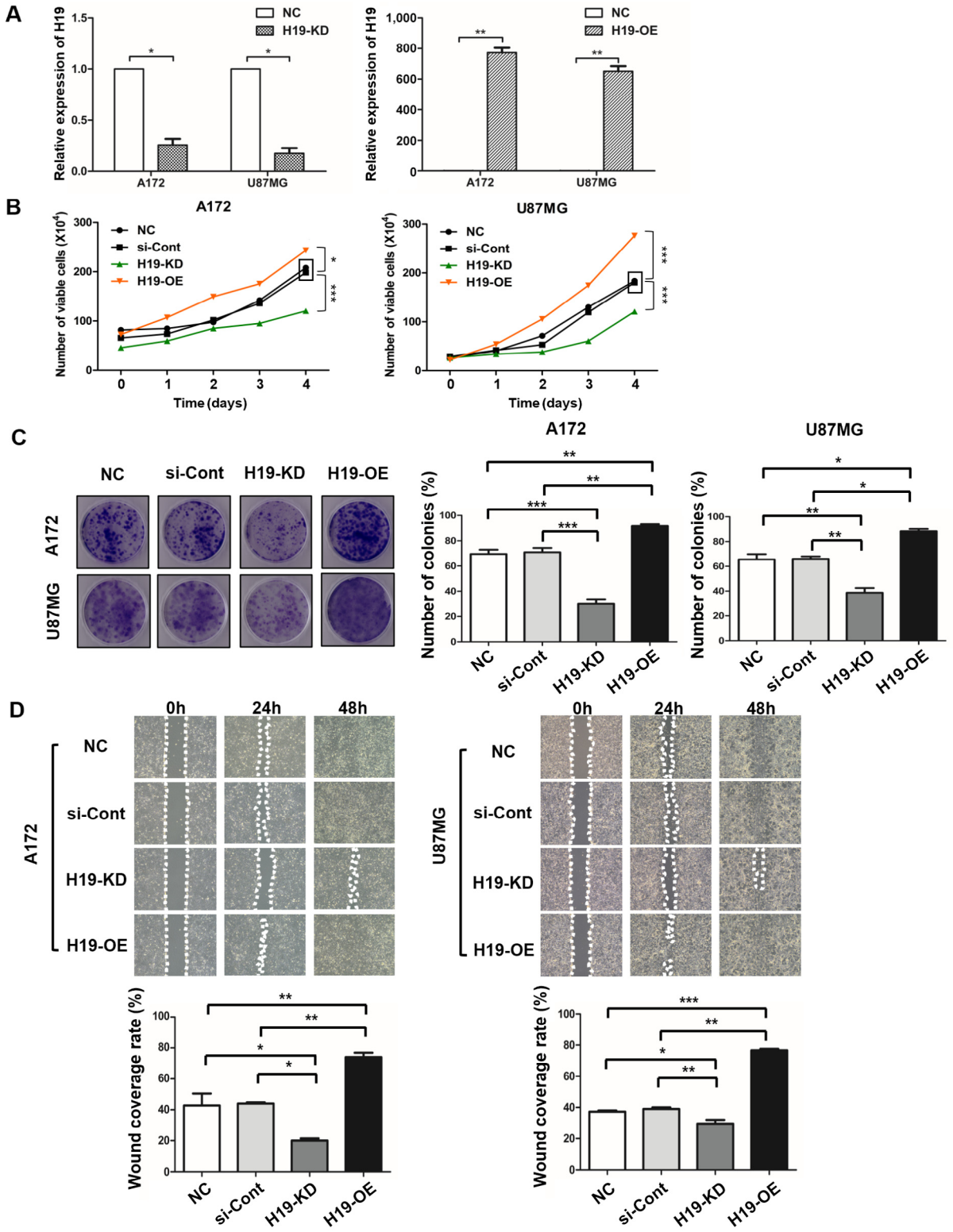

Figure 1. *H19 expression levels and effects of H19 on cell viability and migration. (A) Relative mRNA expression levels of H19 in A172 and U87MG cells were quantified by RT-qPCR. (B) Cell counting assays determined the short-term effects of H19 expression levels on glioma cell lines. (C) Colony formation assays determined the long-term effects of H19 expression levels on glioma cell lines. (D) Wound-healing assays determined the motility of H19 expression levels on glioma cell lines. (*p<0.05, **p<0.01, ***p<0.001). NC: Negative control; si-Cont: scrambled siRNA as control; H19-KD: H19-knockdown; H19-OE: H19-overexpression.*

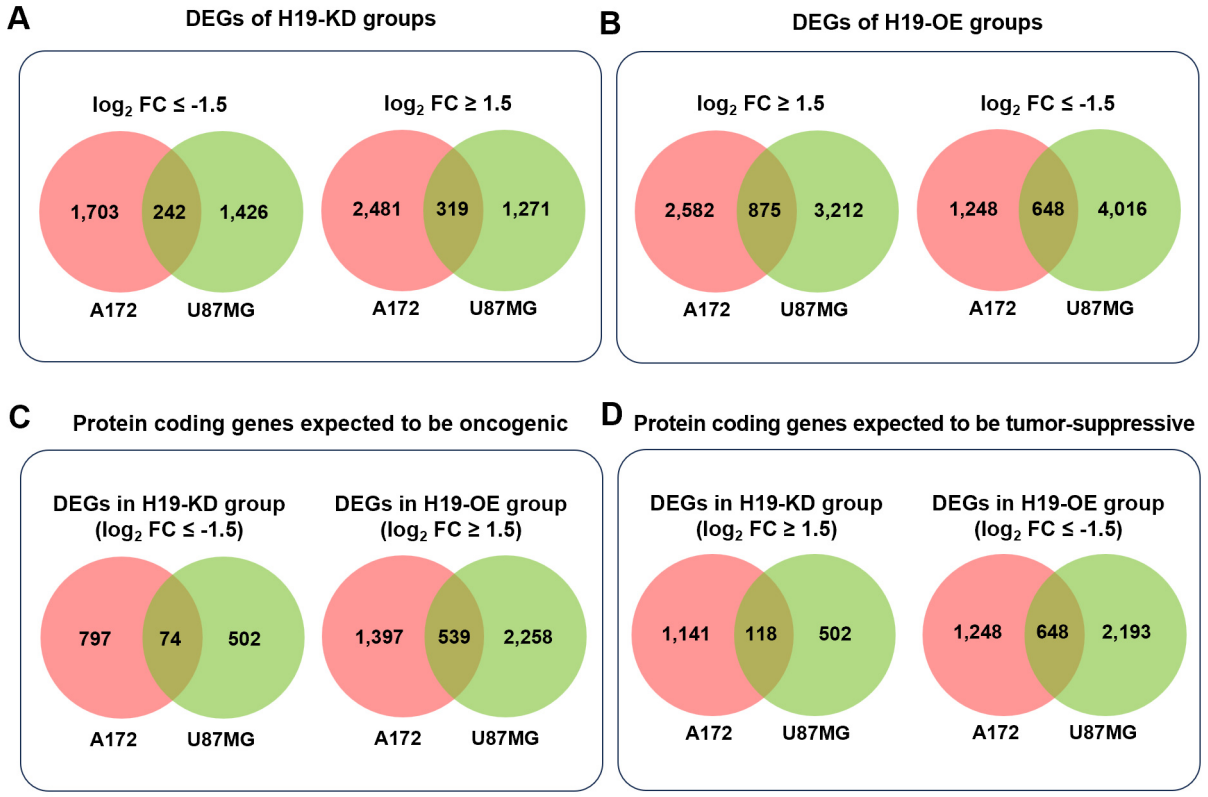

Figure 2. *Common DEGs in the two glioma cell lines regulated by H19. (A) A total of 242 DEGs were down-regulated by ≤–1.5 log_2 fold change in H19-KD group, while 319 DEGs were up-regulated by ≥1.5 log_2 fold change. (B) A total of 875 DEGs were up-regulated by ≥1.5 log_2 fold change in H19-OE group, while 648 DEGs were down-regulated by ≤–1.5 log_2 fold change. (C) A total of 613 protein-coding genes, showing a positive correlation with H19 expression, were expected to be oncogenes. (D) A total of 766 protein-coding genes, showing a negative correlation with H19 expression, were expected to be tumor-suppressive genes. DEG: Differentially expressed gene; H19-KD: H19-knockdown; H19-OE: H19-overexpression.*

Among the DEGs showing positive correlations with H19, 9 DEGs were selected based on existing literatures that have documented oncogenic functions and have been studied in various tumors for their roles in contributing to tumorigenesis (Table II).

Gene expression analyses were performed on these DEGs using GEPIA2 based on TCGA and GTEx databases (Figure 4A). The results revealed elevated expression levels of Annexin A5 (*ANXA5*), C-type lectin domain family 18 member B (*CLEC18B*), RAB42, member RAS oncogene family (*RAB42*), C-X-C motif chemokine ligand 8 (*CXCL8*), 2'-5'-oligoadenylate synthetase like (*OASL*), ubiquitin-specific protease 18 (*USP18*), and CUB domain containing protein 1 (*CDCP1*) in GBM tissues compared to normal tissues. In the case of *ANXA5* and *USP18*, the expression levels were also increased in LGG. Furthermore, survival analyses were performed on these 7 DEGs suggested by the gene expression results, and all showed negative association with overall survival. High expression of each gene, *ANXA5, CLEC18B, RAB42, CXCL8, OASL, USP18*, and *CDCP1*, was associated with high mortality (Figure 4B). Ten DEGs, expected to inhibit tumorigenesis, exhibited tumor-suppressive effects in various cancer types (Table III), and gene expression and survival analyses were conducted on these DEGs. The results showed that the expressions of cold shock domain containing C2 (*CSDC2*) and forkhead box O4 (*FOXO4*) were significantly decreased in GBM (Figure 4C), compared to normal tissue and that low expressions of *CSDC2* and *FOXO4* were associated with high mortality (Figure 4D). Overall, 9 DEGs were selected as target genes based on the results of both bioinformatics analyses. Our results suggest that 7 genes with oncogenic properties (*ANXA5, CLEC18B, RAB42, CXCL8, OASL, USP18*, and *CDCP1*) and 2 with tumor-suppressive properties (*CSDC2* and *FOXO4*) are regulated by H19 and participate in the development of glioma.

Discussion

Previous studies have established the oncogenic significance of H19 in glioma. Although studies have been performed on the pathogenesis and progression of glioma at the molecular level, few studies have addressed H19-related networks.

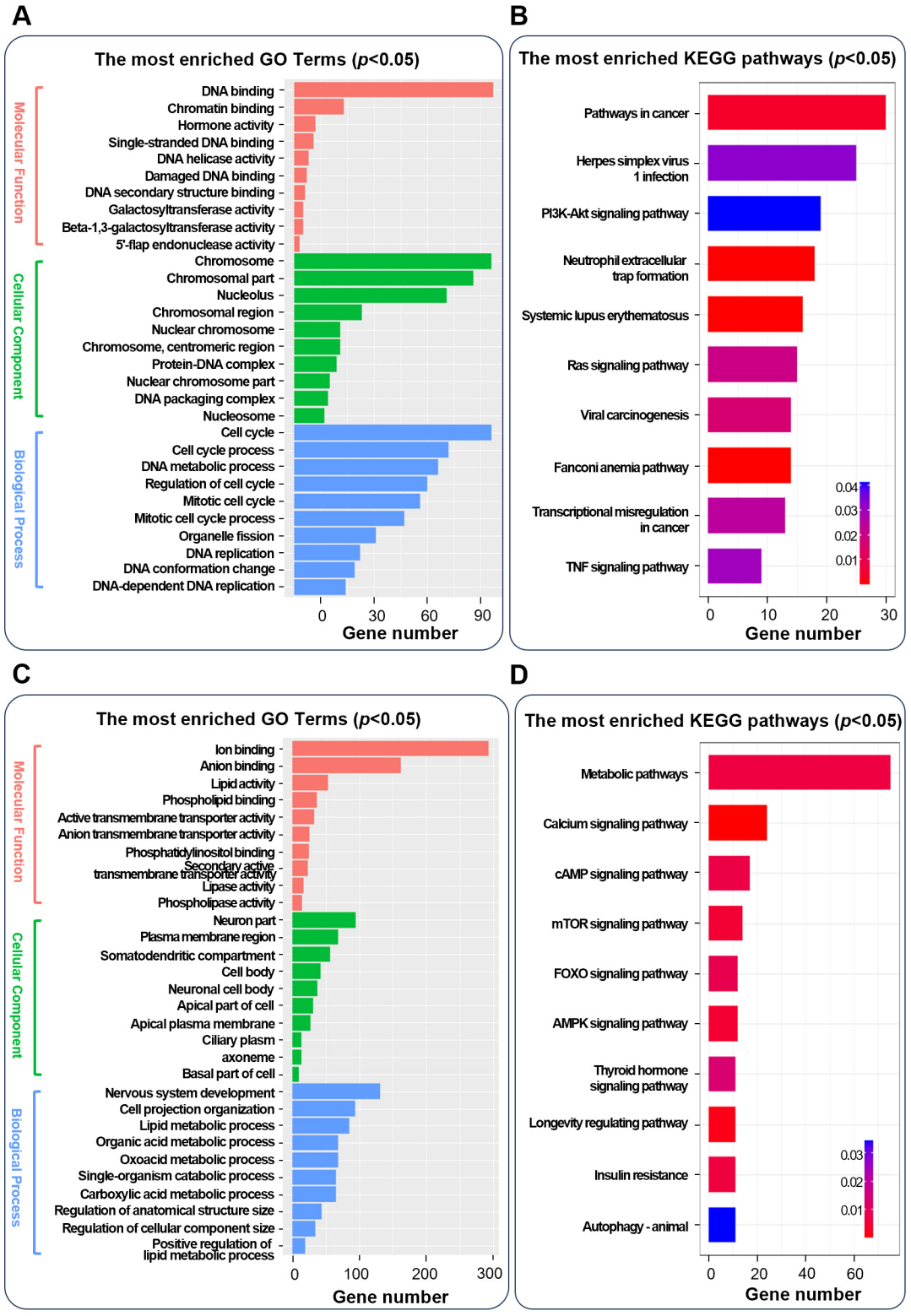

Figure 3. *Functional analyses performed on selected DEGs. (A) The results of GO enrichment analyses highlighted the most enriched GO terms of DEGs putatively promoting tumorigenesis. (B) KEGG pathway enrichment analyses showed the most enriched pathways of DEGs putatively promoting tumorigenesis. (C) GO enrichment analyses displayed the most enriched terms of DEGs presumed to suppress tumorigenesis. (D) KEGG pathway enrichment analyses presented the most enriched pathways of DEGs presumed to suppress tumorigenesis. The color gradient in the KEGG pathway analysis results indicated p-values. DEG: Differentially expressed gene; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genome.*

Table II. *Relative gene expression level of DEGs expected to promote tumorigenesis.*

Group	Gene	$\log_2$ fold change		Oncogenic functions in various tumors	Ref.
		in A172	in U87MG		
H19-KD	*ANXA5*	–2.589248	–2.027261	Facilitating angiogenesis and progression of glioma	(54)
	CLEC18B	–2.421070	–1.776630	Promoting the growth, migration, and invasion of GBM cells	(55)
	RAB42	–2.148033	–1.711191	Promoting cell growth, invasion and tumorigenesis of glioma	(56)
	ETV2	–2.147487	–1.962656	Contributing to the invasion, migration, and EMT process of glioma	(57)
H19-OE	*CXCL8*	115.285663	1.693676	Promoting proliferation and invasion of GBM cells	(58)
	OASL	71.810444	1.635825	Resulting in worse overall survival in breast cancer	(59)
	USP18	29.916867	1.574815	Accelerating GBM cell invasion, migration and EMT	(60)
	BIRC3	15.587837	4.537087	Promoting higher grade glioma and reducing tumor-free survival	(61)
	CDCP1	14.421578	1.999767	Correlating with increasing tumor grade and a poor prognosis in GBM	(62)

ANXA5: Annexin A5; BIRC3: baculoviral IAP repeat containing 3; CDCP1: CUB domain containing protein 1; CLEC18B: C-type lectin domain family 18 member B; RAB42: RAB42, member RAS oncogene family; CXCL8: C-X-C motif chemokine ligand 8; DEG: differentially expressed gene; ETV2: ETS variant transcription factor 2; GBM: glioblastoma; OASL: 2'-5'-oligoadenylate synthetase like; USP18: ubiquitin specific peptidase 18.

Understanding how H19 functions in glioma is essential. Thus, we conducted mRNA expression profiling and bioinformatic analyses on two H19-regulated glioma cell lines to identify genes that interact with H19 and influence glioma. Cell-based assays on A172 and U87MG showed that H19-OE increased cell proliferation, viability, and motility. Total RNA-seq analysis confirmed changes in expression levels of protein-coding and non-coding RNAs including miRNA and lncRNA. Finally, a total of 2,084 DEGs associated with H19 expression were found, of which 1,379 DEGs were protein-coding. Utilizing the DAVID database, GO and KEGG pathway enrichment analyses were performed to functionally analyze these protein-coding DEGs. The results of GO enrichment analyses indicated that DEGs, predicted to promote tumorigenesis, were enriched in 'DNA binding', 'chromatin binding', 'chromosome', and 'cell cycle'. These results are consistent with the previous studies that demonstrated a relationship between chromatin remodeling and cancer therapy highlighting the close association between cell cycle regulation and cancer cell metabolism (74, 75). Additionally, genes involved in cell cycle regulation might be important in tumor progression and aggressiveness in gliomas, playing a crucial role in the regulation of cancer hallmarks (76). KEGG pathway analyses showed significant involvement in the 'Pathways in cancer', 'PI3K-Akt signaling pathway', and 'Ras signaling pathway'. The PI3K-Akt signaling pathway is essential for numerous cellular functions and is abnormally activated in cancers (77), and the Ras signaling pathway is critical for cellular processes including cell growth, survival, and differentiation (78). These findings support that the selected 613 protein-coding DEGs might be associated with cellular proliferation, survival, and motility and suggest that H19 could contribute to glioma malignancy with these genes. In contrast, 766 DEGs, forecasted to suppress tumorigenesis, were identified in GO enrichment analyses with annotations related to 'ion binding', 'neuron part', and 'nervous system development'. These results indicate characteristics associated with stem and progenitor cells rather than correlations between selected DEGs and tumorigenesis. The KEGG pathway analyses on these 766 DEGs identified key pathways including 'cAMP signaling pathway', 'FOXO signaling pathway', 'AMPK signaling pathway', and 'Longevity regulating pathway'. The cAMP signaling pathway is recognized as a significant tumor-suppressive pathway, underscoring its potential role in glioma therapy (79, 80). The FOXO signaling pathway is considered a tumor-suppressive pathway across a wide range of cancers (81). AMPK is a key regulator of cellular energy homeostasis (82), and the AMPK activation suppresses cancer cell growth by inducing cell cycle arrest and inhibiting the oncogenic mTORC1 pathway (83). These signaling pathways have been shown to be associated with energy metabolism, induction of cell death, and cell cycle inhibition, which suggests that down-regulations of these DEGs might be involved in glioma development. In addition, DEGs down-regulated in the H19-KD groups and DEGs up-regulated in the H19-OE groups were predicted to be oncogenes. Conversely, DEGs up-regulated in the H19-KD groups and DEGs down-regulated in the H19-OE group were predicted to be tumor-suppressor genes. Eventually, using bioinformatics analyses, we selected 19 candidate H19-regulated DEGs that function as oncogenes or tumor-suppressor genes. Gene expression analyses and survival analyses were performed on the 19 candidate genes, and comprehensively 9 genes regulated by H19 were found to be involved in glioma development. Thus, further investigations are warranted to elucidate the underlying molecular mechanisms governing the regulations of these 9 genes by H19. *ANXA5, CLEC18B, RAB42, CXCL8, OASL, USP18*, and *CDCP1* levels in LGG and GBM were negatively associated with survival rates. ANXA5 promotes tumor formation and

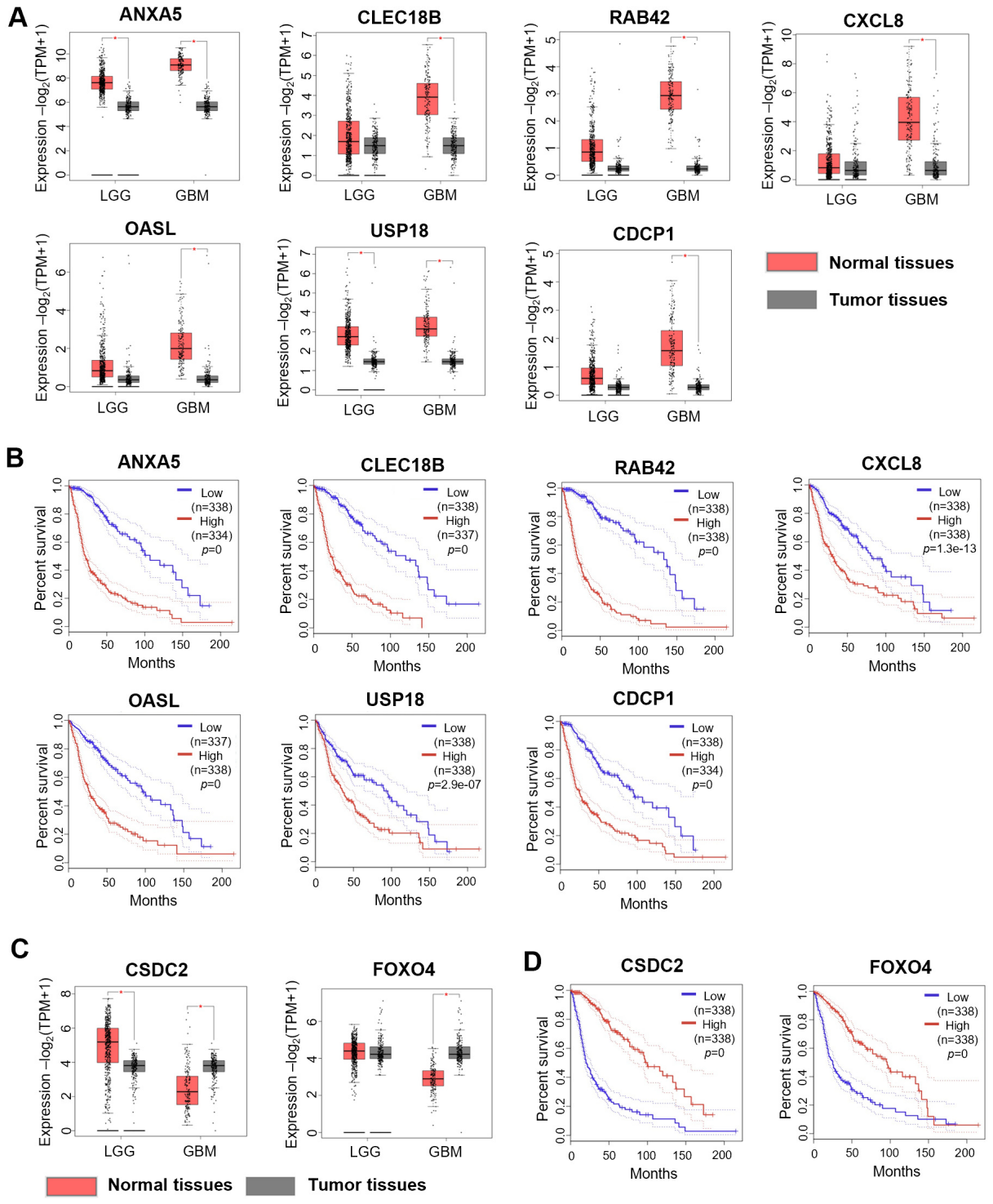

Figure 4. *Gene expression and survival analyses on candidate DEGs in LGG and GBM patient tissue samples. Seven DEGs, ANXA5, CLEC18B, RAB42, CXCL8, OASL, USP18, and CDCP1, predicted to function as oncogenes, (A) showed high expression levels in GBM through gene expression analysis, and (B) were positively correlated with poor overall survival through survival analysis. Two DEGs, CSDC2 and FOXO4, predicted to function as tumor suppressor genes, (C) showed reduced expression levels in GBM through gene expression analysis, and (D) were negatively correlated with poor overall survival through survival analysis. ANXA5: Annexin A5; CDCP1: CUB domain containing protein 1; CLEC18B: C-type lectin domain family 18 member B; CSDC2: cold shock domain containing C2; CXCL8: C-X-C motif chemokine ligand 8; DEG: differentially expressed gene; FOXO4: forkhead box O4; GBM: glioblastoma; LGG: low-grade glioma; OASL: 2'-5'-oligoadenylate synthetase like; RAB42: RAB42, member RAS oncogene family; USP18: ubiquitin specific peptidase 18.*

Table III. *Relative gene expression level of DEGs expected to suppress tumorigenesis.*

Group	Gene	$\log_2$ fold change		Tumor-suppressive functions in various tumors	Ref.
		in A172	in U87MG		
H19-KD	*TNFSF10*	4.7104086	1.719610	Being regarded as an anticancer agent by inducing cancer cell apoptosis	(63)
	ITIH4	1.874714	2.744833	Inhibiting cell proliferation and angiogenesis with KNG1	(64)
	CGN	1.856810	1.535571	Hindering metastasis in colorectal cancer	(65)
	SCUBE2	1.691323	1.780518	Inhibiting glioma cell proliferation, migration, and invasion	(66)
	PGBD3	1.509895	20.535054	Affecting DNA repair and chromatin remodeling	(67)
H19-OE	*CSDC2*	–5.016720	–8.573095	Modulating tumorigenesis in gliomas	(68)
	HSD17B14	–3.470761	–4.1977910	Acting as a tumor-suppressor gene by regulating apoptosis	(69, 70)
	FOXO4	–3.297208	–2.2564431	Suppressing cell growth and malignancy of GBM	(71)
	KLF9	–3.110408	–5.866879	Inhibiting glioma cell proliferation and tumor growth in vivo	(72)
	FGFBP3	–2.9988.00	–2.193251	Being known as protective biomarkers in low-grade glioma	(73)

CGN: cingulin; CSDC2: cold shock domain containing C2; DEG: differentially expressed gene; FGFBP3: fibroblast growth factor binding protein 3; FOXO4: forkhead box O4; GBM: glioblastoma; HSD17B14: hydroxysteroid 17-beta dehydrogenase 14; ITIH4: inter-alpha-trypsin inhibitor heavy chain 4; KLF9: KLF transcription factor 9; PGBD3: piggyBac transposable element derived 3; SCUBE2: signal peptide, CUB domain and EGF like domain containing 2; TNFSF10: TNF superfamily member 10.

angiogenic capacity in glioma (54), while it is known that the growth and progression of glioma depends primarily on angiogenesis (84). Previous studies have indicated that overexpression of H19 promotes GBM cell invasion, angiogenesis, stemness, and tumorigenesis (85), and thus ANXA5 regulation by H19 might facilitate angiogenesis and promote the development and progression of glioma. CLEC18B promotes GBM cell growth, migration, and invasion by increasing Wnt/β-catenin signaling activity (55), and inhibition of H19 suppresses Wnt/β-catenin signaling pathway activation, thereby suppressing the growth, invasion, and migration of glioma cells (27). CLEC18B and H19 play significant roles in glioma malignancy by activating the Wnt/β-catenin signaling pathway, and RAB42 reportedly promotes glioma cell invasion and migration by inducing VEGF signaling (56). Furthermore, H19 has been reported to enhance glioma cell proliferation, migration, invasion, and angiogenesis by increasing the expressions of HIF-1α and VEGF (86). CXCL8 was more highly expressed in GBM than LGG and has been shown to promote cell proliferation and invasion, and activate the NF-κB and Akt pathways (58, 87). H19 also confers temozolomide resistance to glioma cells by activating NF-κB signaling, suggesting H19 and CXCL8 are involved in the same signaling pathway in glioma (88). OASL is up-regulated in astrocytes, which have the capability to transform into glioma cells (89). H19 has been reported to activate astrocytes by modulating the JAK/STAT pathway, which suggests that the up-regulation of OASL in astrocytes is associated with H19 (90). Since OASL is significantly associated with poor overall survival in breast and pancreatic cancers (59, 91, 92) and our results showed that H19 overexpression in A172 and U87MG increased OASL levels, the role of OASL in glioma and its correlation with H19 warrant further investigation. USP18 promotes GBM cell invasion, migration, and EMT by removing ubiquitin from Twist1 and stabilizing the protein (60). In addition to the results of our transcriptome analysis showing USP18 was overexpressed in the H19-OE groups, H19 has been reported to regulate the Twist1 through miR-326 sponge (93), and thus it is possible that USP18 functions as a downstream component of H19. Reportedly, the high expression level of CDCP1 is associated with high tumor grade and poor prognosis in GBM (62). Furthermore, CDCP1 levels are positively correlated with the Wnt signaling pathway (94), and H19, functioning as a ceRNA, has been reported to regulate the Wnt signaling pathway by sponging miR-148 (95). Conversely, survival analyses showed that low levels of *CSDC2* and *FOXO4* (putative tumor-suppressor genes) in GBM tissues were associated with increased mortality. Furthermore, the GEO database showed that CSDC2 expression is down-regulated in GBM, and this down-regulation was reported to be strongly associated with the tumorigenesis of GBM (68). The PI3K/Akt signaling-induced FOXO4 down-regulation has been positively correlated with glioma malignancy, and the up-regulation of FOXO4 inhibited cell proliferation and promoted the apoptosis of GBM cells (71, 96). Furthermore, this signaling pathway has also been reported to be up-regulated by H19 and to promote cell proliferation, invasion, and migration in various cancers (92, 97, 98). Levels of *CSDC2*, a tumor-suppressive gene, were higher in LGG tissues than in normal tissues but significantly lower in GBM tissues. These results suggest that CSDC2 has potential use as a prognostic biomarker in glioma. In addition, *CLEC18B, RAB42, CXCL8, OASL*, and *CDCP1* levels were similar in LGG and normal tissues, but significantly higher in GBM tissues. Conversely, *FOXO4* levels were markedly reduced in GBM compared to LGG tissues. These findings suggest the potential utility of these genes as biomarkers for

distinguishing between LGG and GBM, and that the mechanisms regulated by H19 *via* the selected 9 genes might provide a novel means of diagnosing and treating glioma.

Conclusion

H19 functions as an oncogene by regulating several genes responsible for tumor development and malignancy in glioma cells. *ANXA5, CLEC18B, RAB42, CXCL8, OASL, USP18* and *CDCP1* were predicted to exert oncogenic functions in H19-expressing gliomas, whereas *CSDC2* and *FOXO4* were predicted to play tumor-suppressive roles. Because these 9 genes are influenced by H19 expression, they are expected to interact with H19, but the molecular details underlying these interactions need to be determined experimentally. Nevertheless, total RNA expression profiling and bioinformatics analyses of the effects of H19 in glioma cell lines will enhance our understanding of the role of H19 in glioma development and aid in identifying H19-regulated cancer signaling pathways.

Availability of Data and Materials

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE243116.

Conflicts of Interest

The Authors declare that no conflicts of interest exist regarding the publication of this paper.

Authors' Contributions

YC and JR conducted cell-based experiments and bioinformatics analyses. MI assisted with bioinformatics analyses. WJ curated data and visualized findings. BK and JK were involved in data curation. BY and WK made substantial contributions to the conception and design of the study. YC, JR, BY, and WK authored the original draft. All Authors read and approved the final manuscript and YC, JR, BY and WK confirm the authenticity of all the raw data.

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