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High expression of *HOXA4* in patients with glioma indicates unfavorable clinical outcomes

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ABSTRACT

HOXA4 is a novel oncogene that has been observed in many kinds of tumors, but its role during glioma carcinogenesis and its clinical significance in diagnosing and prognosis human glioma remains unknown. In the present study, the Chinese Glioma Atlas (CGGA)-RNA sequencing database, CGGA microarray, and The Cancer Genome Atlas (TCGA)-RNA seq data from 1674 glioma patients were obtained from online databases and analyzed using quantitative reverse transcription-polymerase chain reaction (RT-qPCR) to detect changes in the expression level of *HOXA4* and characterize the relationship between *HOXA4* and the clinical characteristics and prognosis of patients with glioma. Gene set enrichment analysis (GSEA) was used to reveal how *HOXA4* regulates tumor-related pathways. *HOXA4* mRNA levels in glioma tissue were higher than those in adjacent brain tissue. *HOXA4* expression was also closely related to the clinical and molecular characteristics of gliomas, such as tumor grade and isocitrate dehydrogenase (IDH) mutation. Functional enrichment analysis revealed that *HOXA4* could regulate cancer-related signal pathways, such as Cell cycle, Cell adhesion molecules cams, and JAK/STAT signaling pathway. Results of *in vitro* experiments confirmed that knockdown of HOXA4 blocks the cell cycle pathway and inhibits the proliferation, invasion and chemotherapy resistance in gliomas. We concluded that *HOXA4* was an independent risk factor for glioma and may have clinical diagnostic potential. Meanwhile, our findings revealed that *HOXA4* could be used as a biomarker for glioma diagnosis and treatment.

Introduction

Glioma is the most common intracranial malignant tumor caused by neural stem cells or neural precursor cells and is associated with a high postoperative recurrence rate and resistance to chemotherapy. The annual incidence is about 4.5/100,000 [1,2]. According to the 2007 World Health Organization (WHO) histopathological standards, gliomas can be classified according to various types and malignancy grades (I–IV) [3]. With increasing tumor grade, the invasion and malignancy of the tumor cells gradually increase [4]. To improve the prognosis outcomes of patients with glioma, comprehensive treatments like neurosurgical resection, adjuvant radiotherapy, and alkylating agent temozolomide chemotherapy have been used [5]. However, the clinical prognosis of patients with glioma is still unsatisfactory [6]. The 5-year survival rate of patients with low-grade glioma is 30–70%, while the median survival time of patients with the most malignant glioblastoma is only 9–12 months, and the 5-year survival rate is less than 5% [7]. This is mainly due to the complicated,

poorly understood pathological mechanism of glioma and the lack of effective therapeutic targets. The search for a reliable biomarker for the diagnosis and prognosis of glioma is therefore of great urgency. Previous reports have confirmed that the *HOX* gene family plays a vital regulatory role in the evolution of cancers and is strongly associated with prognosis [8,9].

HOXA, HOXB, HOXC, and *HOXD* constitute the *HOX* gene family and are located in different chromosomal regions [10]. The *HOXA* gene family encodes proteins that contain the DNA-binding homeobox motif and controls the early patterns of embryo segmentation in addition to later developmental events [11]. Several studies have shown that the *HOXA* gene family is associated with various cancer types [12,13]. For instance, Li et al. reported that overexpression of *HOXA10, HOXA11,* and *HOXA13* serves as a predictive biomarker of unfavorable survival in patients with laryngeal squamous cell cancer [14]. As a key member of the a subfamily, *HOXA4* is widely involved in various physiological and pathological processes in organisms through many well-known signaling pathways [9,15,16]. *HOXA4* plays an important role in cell reproduction, differentiation, apoptosis, and signal transduction [17]. Current studies have shown that abnormal expression of *HOXA4* plays an important role in the pathogenesis and affects the prognosis and recurrence of various cancers [18–20]. For instance, *HOXA4* can inhibit the proliferation and gefitinib resistance of non-small cell lung cancer by regulating miR-138 [21]. Accumulated evidence has indicated the abnormal expression of *HOXA4* in cancers could make contributions to carcinogenesis. Nevertheless, no study has previously reported a significant association between *HOXA4* expression and prognosis of human glioma.

In the present study, we comprehensively analyzed the molecular pattern and clinical relevance of *HOXA4* in glioma patients. We evaluated its expression, prognostic value, and biological functions in a large cohort of glioma samples. Abnormally high expression of *HOXA4* was observed in glioma tissue using quantitative reverse transcription-polymerase chain reaction (RT-qPCR) and immunohistochemistry (IHC) at the mRNA and protein levels, respectively. Our results suggested that a significant association between *HOXA4* expression and poor prognosis in glioma patients. Also, experimental results suggest that *HOXA4* is involved in regulating the malignant characteristics of gliomas. Furthermore, *HOXA4* expression may be used to inform the diagnosis and treatment of human glioma as an independent risk factor.

Materials and methods

Data collection

A total of 1674 patients with glioma were collected from the Chinese Glioma Atlas (CGGA, http://www.cgga.org.cn/help.jsp) [22] and The Cancer Genome Atlas (TCGA, https://portal.gdc.cancer.gov/) [23], including: CGGA-RNA sequencing database (n = 749), CGGA- microarray database (n = 268), and TCGA-RNA seq (n = 657). Table S1, Table S2, and Table S3 provide the patients' clinical information in these three databases. The microarray dataset GSE4290 was downloaded from the Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/geo/) database [24], and the Limma package in R software (v3.6.1) was used to evaluate the differential expression of *HOXA4* in glioma tissues and non-tumor tissues. The Gene Expression Profiling Interactive Analysis (GEPIA, http://gepia.cancer-pku.cn) contains expression data from tumor and non-tumor samples [25]. The *HOXA4* glioma expression level data was obtained from GEPIA, while protein localization and expression data from human tissues and cells were obtained from the Human Protein Atlas (HPA, https://www.proteinatlas.org/) [26]. The HPA contains validated antibodies for mapping the entire human proteome with immunohistochemistry (IHC) and immunocytochemistry (ICC) [27]. Then, we verified the *HOXA4* expression in glioma tissue by using reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Tumor and adjacent brain tissue from five glioma patients were obtained from Henan Provincial People's Hospital during surgery and stored in liquid nitrogen. The clinical

characteristics were presented in Table S4. The use of patient samples complied with the Declaration of Helsinki. The study protocol was approved by the ethics committee of Henan Provincial People's hospital (2020, ethical review No. 107).

Gene set enrichment analysis (GSEA) of HOXA4

GSEA analysis can be used to determine if there are statistically significant differences in a set of predefined genes between two biological states. We used GSEA to determine whether the differences in the expression levels of the predefined genes between the high-level and low-level HOXA4 expression groups were statistically significant. Each analysis involved 1000 genome permutations. We then subjected the dataset to enrichment analysis by using the KEGG database.

Cell lines and cell culture

The human glioma cell line (U251) and the normal control cell (Human astrocyte, HA) were purchased from Procell Life Science & Technology Co. Ltd (Wuhan, China). Both cell lines were maintained in DMEM medium supplemented with 10% FBS(Gibco) and 1% penicillin/streptomycin (Procell, Wuhan, China). All cells were cultivated in an incubator with constant temperature containing a 5% CO2 atmosphere at 37°C.

Cell transfection

The small interfering RNA (siRNA) was constructed by GenePharma (GenePharma, Shanghai, China). Table 1 shows all the sequences of HOXA4 siRNA and negative control (NC) siRNA. According to the instructions, the U251 cells in the logarithmic growth phase were digested by 0.25% pancreatin and evenly

Table 1. RNA oligo sequence of siRNA.

siRNA	sequence (5'-3')
siRNA-NC (sense)	UUCUCCGAACGUGUCACGUTT
siRNA-NC (antisense)	ACGUGACACGUUCGGAGAATT
siRNA-1 (sense)	GCCUCUCGCCGGAAGAGUUTT
siRNA-1 (antisense)	AACUCUUCCGGCGAGAGGCTT
siRNA-2 (sense)	GGACCUCUUUCGGGCAGUUTT
siRNA-2 (antisense)	AACUGCCCGAAAGAGGUCCTT
siRNA-3 (sense)	GCAGAGGGACUACGUGAUUTT
siRNA-3 (antisense)	AAUCACGUAGUCCCUCUGCTT

placed on a 6-well plate. After 60–70% confluence, the cells were transfected with HOXA4-siRNA and NC-siRNA (100 pmol/l) using siRNA-Mate (GenePharma, Shanghai, China). The transfection efficiency was measured by RT-qPCR analysis 48 h after transfection. The siRNA with the best transfection efficiency was used for subsequent experiments.

RNA isolation and RT-qPCR analysis

Use RT-qPCR to detect HOXA4 expression in glioma tissues and cells. First, total RNA was isolated using the TRI-Reagent (Sigma, USA), according to the manufacturer's instructions. RNA quality was estimated using the NanoDrop One Spectrophotometer (Thermo Fisher Scientific, USA). Total RNA was reverse transcribed into cDNA using a Transcriptor First Strand cDNA Synthesis Kit (Roche, USA), as per the manufacturer's instructions. RT-qPCR was performed using the FastStart Universal SYBR Green Master (ROX) kit (Roche, Germany), according to the manufacturer's instructions. The RT-qPCR results were analyzed using QuantStudio software (Thermo Fisher Scientific, USA), with GADPH as an internal reference. The primer sequence used was manifested as follows: GAPDH forward 5'-CAAGGTCATCCATGACAACTTTG-3' and GAPDH reverse 5'-GTCCACCACCCTGTTGCTGTAG-3'; HOXA4 forward 5'-ATAACGGAGGGGAGCCTAAG-3' and HOXA4 reverse 5'-GCTCAGACAAACAGAGCGTG –3'.

CCK8 assay

CCK8 assay (Dojindo, Japan) was used to detect cell proliferation and chemotherapeutic resistance in U251. A total of 24 h post-transfection, the transfected U251 cells were seeded into 96-well plates and used CCK8 to detect cell proliferation conditions after culturing for 96 hours. The detection steps are as follows: removing the medium, then adding 10 μl of CCK-8 reagent and 90ul DMSO medium to each well, followed by incubation for an additional 4 h. Finally, a spectrophotometer was used to detect the absorbance value (450 nm) to compare the cell proliferation speed of each group.

Colony formation analysis

The transfected U251 cells were seeded into a 6-well plate (200 cells/well) and cultured in a complete culture medium for 2 weeks. When about 50 cell clusters were seen under the microscope, the 6-well plate was taken out from the incubator. The cell colonies were washed with PBS, then fixed with 4% paraformaldehyde at room temperature for 30 minutes and stained with crystal violet for 10 minutes. Subsequently, washing cell colonies with ultrapure water and drying upside down. After taking pictures, using ImageJ software (Version 1.52) for subsequent analysis.

Wound healing assay

U251 cells to make the single-cell suspension, then cells were seeded in a 6 well plate at 4×10^5/well and incubated in an incubator at 37°C with 5% CO_2. When cells grew to 90% confluence, a linear scratch in the cell monolayer was made with a 200 µl sterile pipette and then washed 3 times with 1× PBS to remove detached cells. After that, the scratch width was observed and photographed under the microscope for 0 h and 48 h, respectively, and measured by using ImageJ software (Version 1.52).

Flow cytometry analyses for cell cycle

U251 cells transfected with siRNA were harvested and fixed in 70% ethanol overnight at −20°C. Next, cells were resuspended using 50 µg/µL propidium iodide (PI) and incubated with 0.1 µg/µL RNase for 30 min, and then applied to flow cytometry.

Western blotting

U251 cells treated with *HOXA4*-siRNA and NC-siRNA were collected and lysed, and total proteins were extracted for electrophoretic analysis. The proteins were then transferred to PVDF membranes, and skim milk was used to seal the membrane. Specific antibodies (CDK2, Cat 10,122-1-AP, Proteintech, China; CDK4, Cat 11,026-1-AP, Proteintech, China; Cyclin

E1, Cat 11,554-1-AP, Proteintech, China; β-actin, Cat 66,009-1-Ig, Proteintech, China) are used to incubate the membrane at 4°C overnight. After further incubation with HRP-conjugated antibodies, protein bands were shown and recorded.

Statistical analysis

R software (v.3.6.1 version) and GraphPad Prism (v8.0.2) was used to process data and perform statistical analysis. The association between clinical characteristics and gene expression was analyzed by the Wilcox test and the Kruskal-Wallace test. We used the Kaplan-Meier method and Cox regression analysis to analyze the relationship between *HOXA4* expression level and survival rates. P < 0.05 was statistically significant.

Results

Abnormal expression of HOXA4 in tumor tissues

The Gene Expression Profiling Interactive Analysis (GEPIA) database contains gene expression data for different human tumors. We searched for *HOXA4* data and found that the *HOXA4* expression level increased significantly in tumors such as glioblastoma multiforme, pancreatic adenocarcinoma, thymoma, and kidney renal clear cell carcinoma. In contrast, its expression level decreased in tumors such as invasive breast carcinoma, esophageal carcinoma, colon adenocarcinoma, kidney chromophobe, lung adenocarcinoma, lung squamous cell carcinoma, rectum adenocarcinoma, thyroid carcinoma, and uterine corpus endometrial carcinoma. The expression level of *HOXA4* in glioblastoma (GBM) was higher than that in non-tumor tissues (Figure 1(a)).

To further investigate the expression level and pathological role of *HOXA4* in glioma tissue, we obtained a dataset from the Gene Expression Omnibus (GEO) database of GSE4290 glioma expression levels in 77 glioma tissue specimens and 23 standard brain tissue specimens. According to this dataset, the expression level of *HOXA4* was significantly increased in glioma tissue relative to that in adjacent brain tissue (Figure 1(b)). We verified this by measuring the expression level of *HOXA4* in five gliomas and five non-tumor tissue samples by using

a

b

c
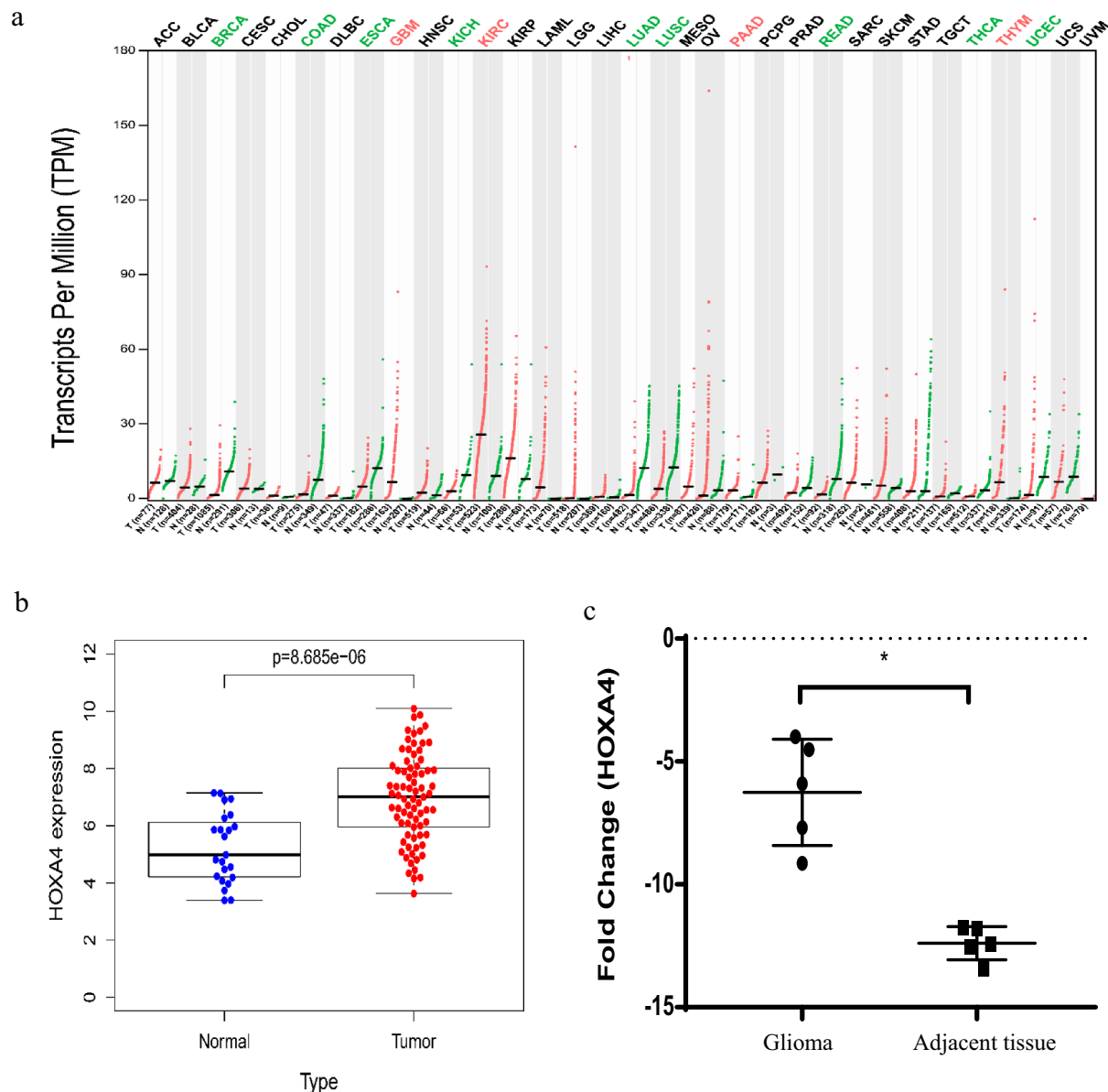
Figure 1. (a) Expression levels of HOXA4 in various human tumor tissue samples. The color of the tumor type represents the level of HOXA4 expression in the tumor: red denotes high expression, while green denotes low expression. (b) Seventy-seven glioma tissue specimens were compared with twenty-three standard brain tissue specimens obtained from the GSE4290 dataset. The expression level of HOXA4 in glioma tissue was significantly higher than that in normal brain tissue. (c) Results of RT-qPCR comparison of HOXA4 levels in five glioma tissue samples and five adjacent brain tissue samples. The RT-qPCR results verified that HOXA4 was expressed at higher levels in glioma tissue than in normal tissue.

RT-qPCR (Figure 1(c)). Our RT-qPCR analysis results confirmed that the expression of *HOXA4* was higher in glioma tissue than that in normal brain tissue, but the function of *HOXA4* in gliomas remains unknown.

Increased expression of HOXA4 led to a reduction in the overall survival of glioma patients

To determine the clinical significance of *HOXA4* expression regarding the prognosis of glioma patients, we performed a Kaplan-Meier analysis of 1672 patients with glioma. By analyzing the data from three databases, we determined that the overall survival (OS) of patients with elevated *HOXA4* expression was considerably lower than that of patients with low expression of *HOXA4* (Figure 2(a, e, i)) but that the relationship between the high expression level of *HOXA4* and survival was different for patients with different grades of glioma. In the CGGA RNA-seq dataset, for

patients with WHO II, III, and IV glioma, a high *HOXA4* expression level was associated with a shorter survival time (Figure 2(b–d)). In the TCGA RNA-seq dataset, a high *HOXA4* expression level was also associated with reduced survival time in patients with WHO III glioma. However, there was no association with the survival time of patients with WHO II and WHO IV glioma (figfigure 2(f–h)). In the CGGA microarray dataset, although an elevated *HOXA4* expression level was associated with a reduced survival time of WHO II and III glioma patients, its association with the survival time of WHO IV glioma patients were not statistically significant (Figure 2(j–l)). This suggested that the overexpression of *HOXA4* was closely linked to poor prognosis in glioma patients.

However, the relationship between *HOXA4* and clinicopathological features remains unclear.

Univariate and multivariate analysis results

Univariate analysis with Cox regression revealed that the expression of *HOXA4* as a categorical dependent variable was linked to unfavorable clinical outcomes. In the CGGA RNA-seq dataset, a high level of *HOXA4* expression in glioma patients was significantly linked to poor prognosis ($P < 0.001$, [HR] = 1.537 (95% confidence interval (CI) [1.449–1.630])), older age ($P < 0.001$, [HR] = 1.624 (95% CI [1.345–1.960])), high histology ($P < 0.001$, [HR] = 4.487 (95% CI [3.695–5.449])), PRS type ($P < 0.001$, [HR] = 2.123 (95%
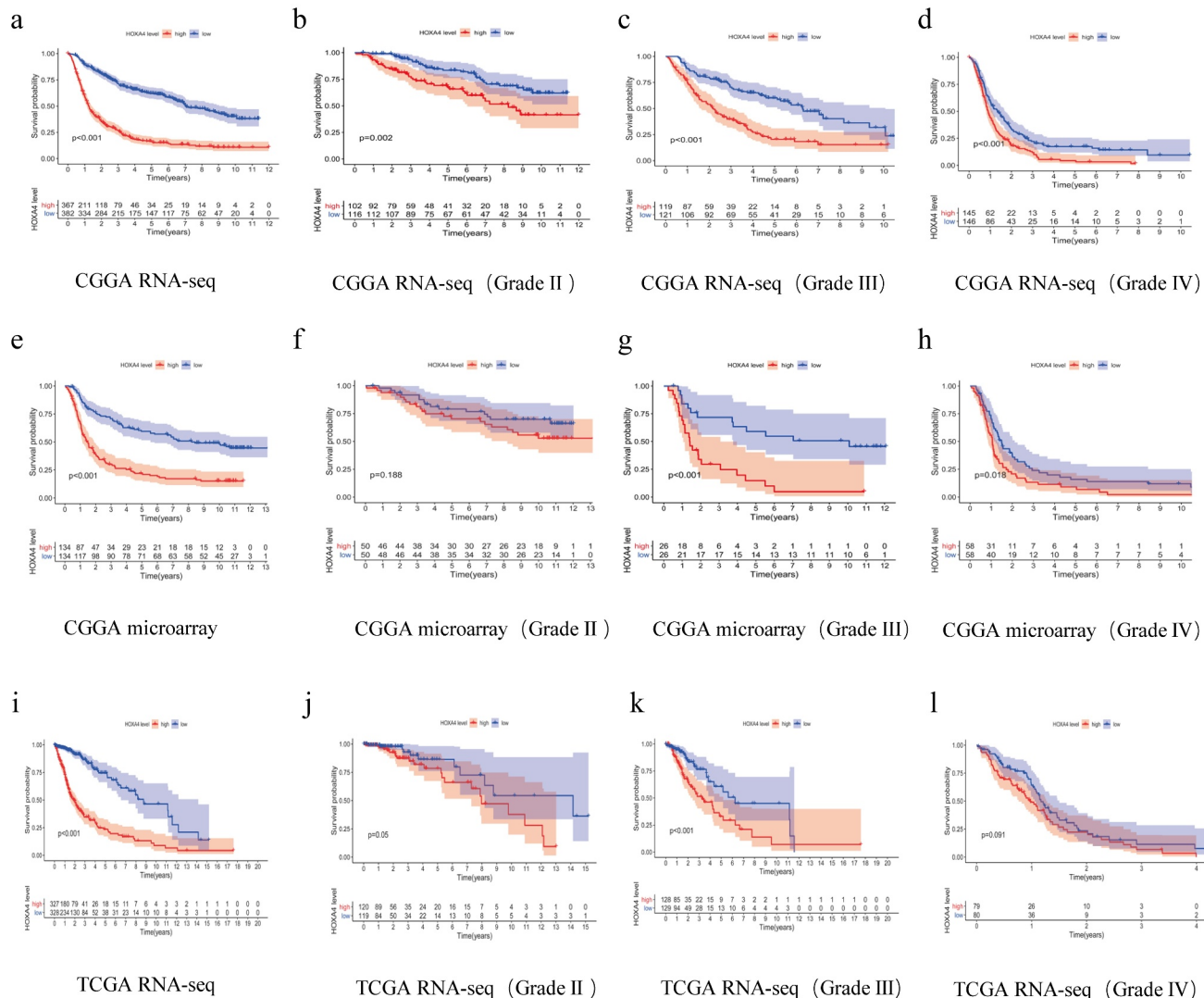
Figure 2. Impact of HOXA4 expression on the overall survival of glioma patients. Data are from the CGGA RNA-seq dataset (a–d), CGGA microarray dataset (e–h), and TCGA RNA-seq dataset (i–l).

CI [1.818–2.478])), high grade (P < 0.001, [HR] = 2.883 (95% CI [2.526–3.291])), 1p19q-codeletion (P < 0.001, [HR] = 0. 231 (95% CI [0.169–0.315])), and IDH mutation (P < 0.001, [HR] = 0.317 (95% CI [0.262–0.384])) (Figure 3 (a)). In the CGGA microarray dataset, a high *HOXA4* expression level in glioma patients was closely associated with poor prognosis (P < 0.001,

[HR] = 1.301 (95% CI [1.225–1.381])), older age (P < 0.001, [HR] = 1.736 (95% CI [1.283–2.349])), high histology (P < 0.001, [HR] = 4.437 (95% CI [3.235–6.086])), PRS type (P < 0.001, [HR] = 2.042 (95% CI [1.511–2.478])), high tumor grade (P < 0.001, [HR] = 2.567 (95% CI [2.125–3.100])), and IDH mutation (P < 0.001, [HR] = 0.423 (95% CI [0.309–0.579])) (Figure 3
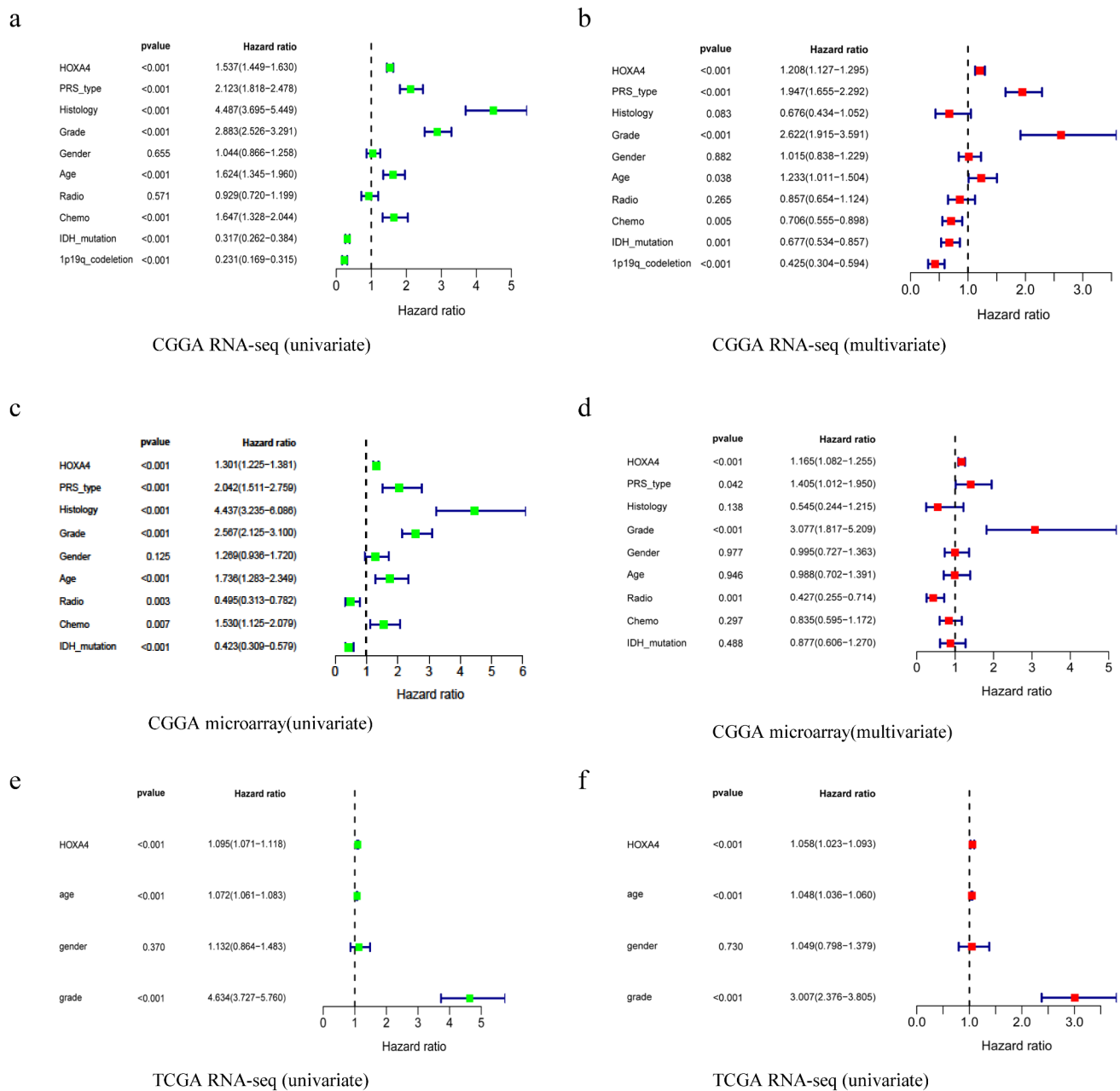
Figure 3. Results of univariate and multivariate analysis of clinical characteristics and HOXA4 expression data using a Cox regression model: results of (a) univariate analysis of the CGGA RNA-seq database, (b) multivariate analysis of the CGGA RNA-seq database, (c) univariate analysis of the CGGA microarray database, (d) multivariate analysis of the CGGA microarray database, (e) univariate analysis of the TCGA RNA-seq database, and (f) multivariate analysis of the TCGA RNA-seq database. Overall, higher HOXA4 expression levels were associated with poor clinical prognosis and survival in glioma patients.

(c)). In the TCGA RNA-seq dataset, a high level of *HOXA4* expression in glioma patients was significantly associated with poor prognosis (P < 0.001, [HR] = 1.301 (95% CI [1.225–1.381])), older age (P < 0.001, [HR] = 1.736 (95% CI [1.283–2.349])), and high tumor grade (P < 0.001, [HR] = 2.567 (95% CI [2.125–3.100])) (Figure 3(e)).

We next performed multivariate analysis with a Cox regression model. In the CGGA RNA-seq dataset, a high level of *HOXA4* expression in glioma patients was closely linked with poor prognosis (P < 0.001, HR = 1.208 (95% CI [1.127–1.295])), PRS type (P < 0.001, HR = 1.947 (95% CI [1.655–2.292])), high grade (P < 0.001, HR = 2.622 (95% CI [1.915–3.591])), and 1p19q-codeletion (P < 0.001, HR = 0.425 (95% CI [0.304–0.594])) (Figure 3(b)). In the CGGA microarray dataset, a high level of *HOXA4* expression in glioma patients was significantly linked with poor prognosis (P < 0.001, HR = 1.165 (95% CI [1.082–1.225])) and patients with high tumor grade (P < 0.001, HR = 3.077 (95% CI [1.817–5.209])) (Figure 3(d)). In the TCGA RNA-seq database, a high level of *HOXA4* expression in glioma patients was linked to poor prognosis (P < 0.001, HR = 1.058 (95% CI [1.023–1.093])) and older age (P < 0.001, HR = 3.007 (95% CI [2.376–3.805])) (figure 3(f)). These results suggested that a high *HOXA4* expression level might be a significant factor associated with poor prognosis in glioma patients.

Clinical diagnostic value of HOXA4

To validate the results of database-level analysis of *HOXA4* expression in glioma tissue, ROC curves were generated for *HOXA4* expression in gliomas of all grades. The three-year survival area under the curve (AUC) for patients with *HOXA4* expression was 0.769 in the CGGA RNA-seq dataset (Figure 4(a)), 0.784 in the CGGA microarray dataset (Figure 4(e)), and 0.838 in the TCGA RNA-seq database (Figure 4(i)). This indicated that the expression level of *HOXA4* had diagnostic value that may be valuable in predicting the prognosis of patients with glioma. Interestingly, in the CGGA RNA-seq and CGGA microarray datasets, *HOXA4* expression had diagnostic potential for WHO II gliomas (Figure 4(b, f)) and statistically significant

diagnostic potential for WHO III gliomas in all three datasets (Figure 4(c, g, k)). As shown in Figure 4(d,l), the abnormally high expression level of *HOXA4* observed in the CGGA RNA-seq, and TCGA RNA-seq databases had a credible predictive value for the prognosis of WHO III glioma patients at 5 years.

Correlation between HOXA4 expression and clinical characteristics in glioma patients

Glioma tissue with associated *HOXA4* expression data from the CGGA RNA-seq, CGGA microarray, and TCGA RNA-seq datasets were analyzed (Figure 5(a–g)). In the CGGA RNA-seq dataset, the expression level of *HOXA4* was strongly associated with the tumor grade (P < 0.001), age (P < 0.001), histological type (P < 0.001), chemotherapy status (P < 0.001), 1p19q-codeletion status (P < 0.001), PRS type (P < 0.001), and IDH mutation status (P < 0.001) of the patients. In the CGGA microarray dataset, the expression level of *HOXA4* was strongly associated with the tumor grade (P < 0.001), age (P < 0.001), histological type (P < 0.001), PRS type (P = 0.021), and IDH mutation status (P < 0.001) of the patients. In the TCGA RNA-seq dataset, the expression level of *HOXA4* was strongly correlated with the tumor grade (P < 0.001) and age (P < 0.001) of the patients.

HOXA4-related cellular signaling pathways

Gene set enrichment analysis (GSEA) was used to identify glioma-related signaling pathways in the high and low *HOXA4* expression level CGGA-RNA seq data. This analysis revealed significant differences (NOM P-value <0.05 and FDR q-value <0.25) in the enrichment of the MSigDB collection (c2.cp.biocarta and h.all. v6.1. symbols). As shown in Figure 6 and Table 2, Cell cycle, Cell adhesion molecules cams, and Jak stat signaling pathway were all associated with the high HOXA4 expression level phenotype. The above pathways are closely related to cancer, indicating that *HOXA4* may participate in the carcinogenesis and progression of glioma through these three tumor-related signaling pathways.
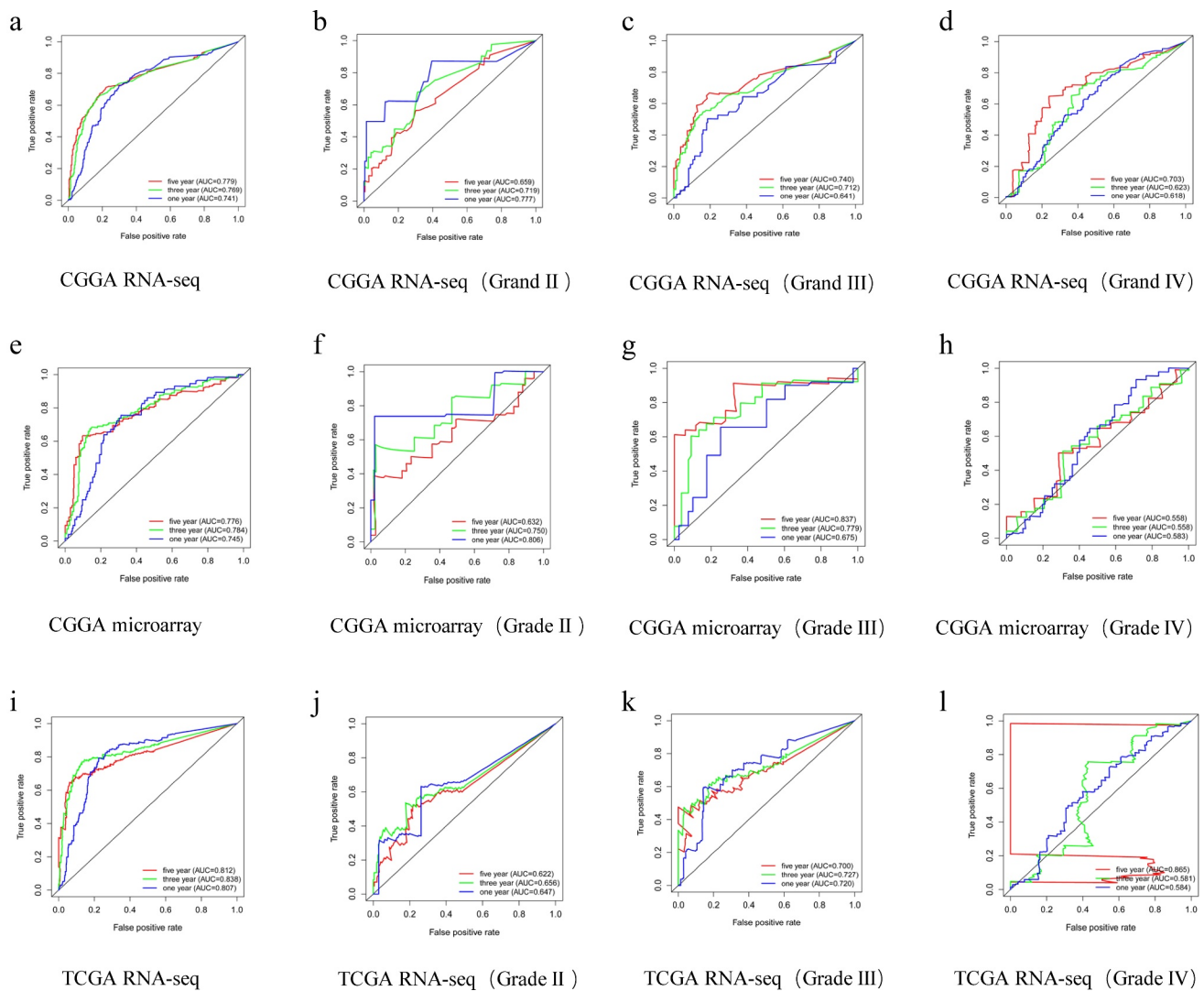
Figure 4. Diagnostic potential of HOXA4 expression in glioma. Results of analyzing the CGGA RNA-seq dataset (a-d), CGGA microarray dataset (e-h), and TCGA RNA-seq dataset (i-l).

Increased HOXA4 protein expression in glioma tissues

The Human Protein Atlas (HPA) provides a map of the distribution and relative abundance of proteins in normal brain tissue, low-grade glioma tissue, and high-grade glioma tissue. We divided this data into male v and female (Figure 7(d,e)) groups. Compared with non-tumor tissues, *HOXA4* protein expression in glioma tissues was upregulated, and its expression in high-grade glioma tissue was higher than that in lower-grade glioma tissue.

Verification of the bioinformatics analysis results

The above bioinformatics analysis results fully reveal that overexpression of *HOXA4* could be used as an independent risk factor leading to poor prognosis in patients with glioma. To explore the vital role of *HOXA4* in glioma more scientifically and rigorously, we further conducted in vitro experiments to validate our analysis results. First, we used RT-qPCR to detect the expression level of *HOXA4* in the glioma cell line (U251) and corresponding normal cells (HA). As presented in Figure 8(a), the result showed that the *HOXA4* expression level was significantly elevated in glioma cell lines. Then, we used siRNA to knock down the *HOXA4* in U251 cells to evaluate the effect of U251 on the malignant biological behavior of glioma cells. Three siRNA fragments named siRNA-1, siRNA-2, and siRNA-3 were used, and siRNA-2 was chosen for subsequent experiments (Figure 8(b)). The results of the CCK8 assay showed that the downregulation of *HOXA4* could inhibit cell proliferation ability and
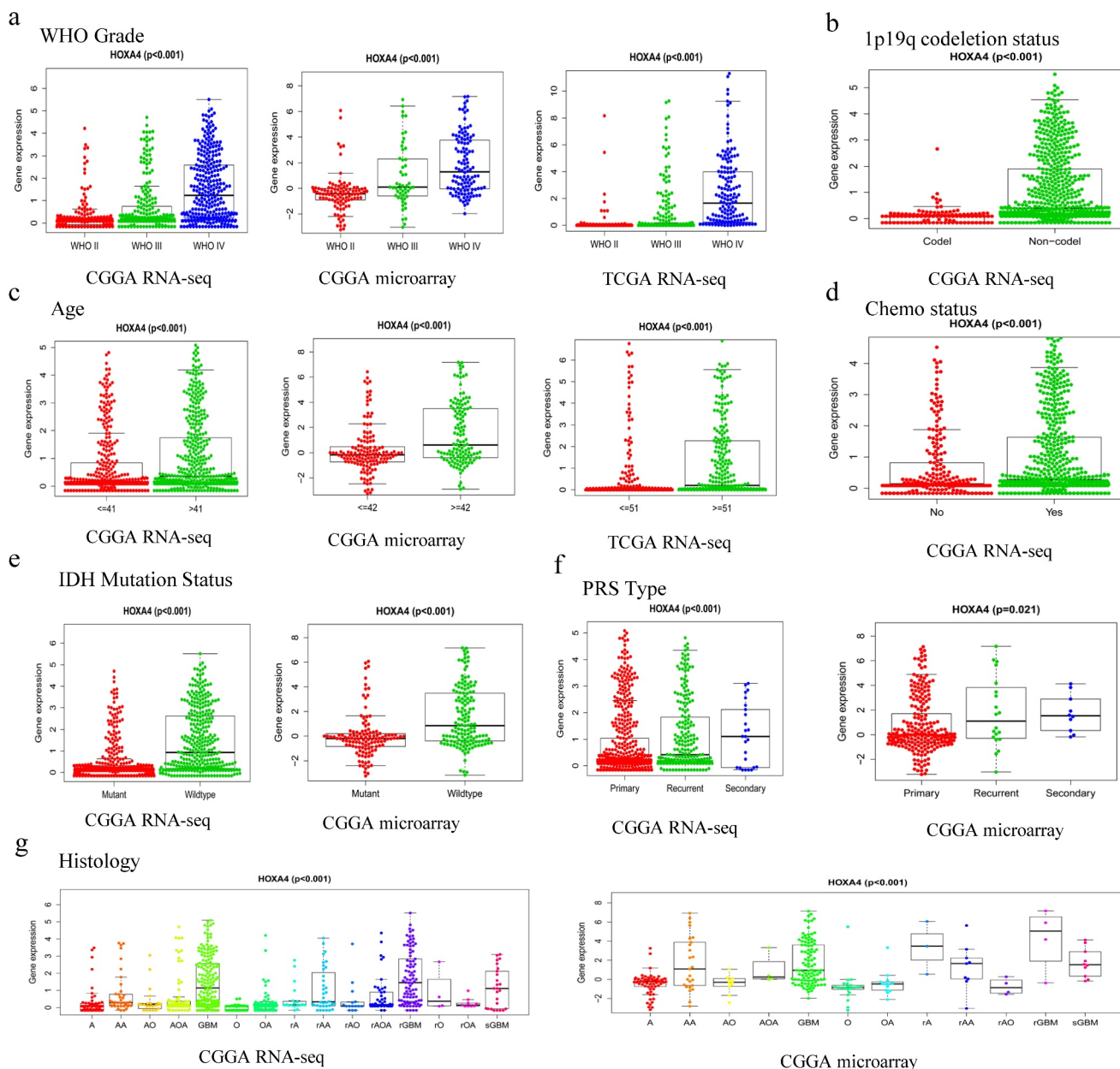
Figure 5. The relationship between the expression level of HOXA4 and various clinicopathological characteristics according to data obtained from the CGGA-RNA sequencing, CGGA microarray, and TCGA-RNA seq datasets. (a) Tumor grade, (b) 1p19q_codeletion status, (c) age, (d) chemotherapy status, (e) IDH mutation status, (f) PRS type, (g) Histology. A: Astrocytoma; AA: Anaplastic astrocytoma; AO: Anaplastic oligodendroglioma; AOA: Anaplastic oligoastrocytoma; GBM: Glioblastoma; O: Oligodendroglioma; OA: Oligoastrocytoma; rA: Relapse astrocytoma; rAA: Relapse anaplastic astrocytoma; rAO: Relapse anaplastic oligodendroglioma; rAOA: Relapse anaplastic oligoastrocytoma; rGBM: Relapse glioblastoma; rO: Relapse oligodendroglioma; rOA: Relapse oligoastrocytoma; sGBM: Secondary relapse glioblastoma.

chemotherapeutic resistance in U251 cells (Figure 8 (c)). In addition, the results of the colony formation assay also confirmed that the cell proliferation ability decreased obviously after the reduction of *HOXA4* expression, which was consistent with the CCK8 assay results (Figure 8(d,e)). Finally, the wound

healing experiment results demonstrated that *HOXA4* expression decreased could significantly inhibit the migration ability of U251 cells compared with the negative control group (figure 8(f,g)). Taken together, the results of in vitro experiments indicate that *HOXA4* overexpression may lead to poor
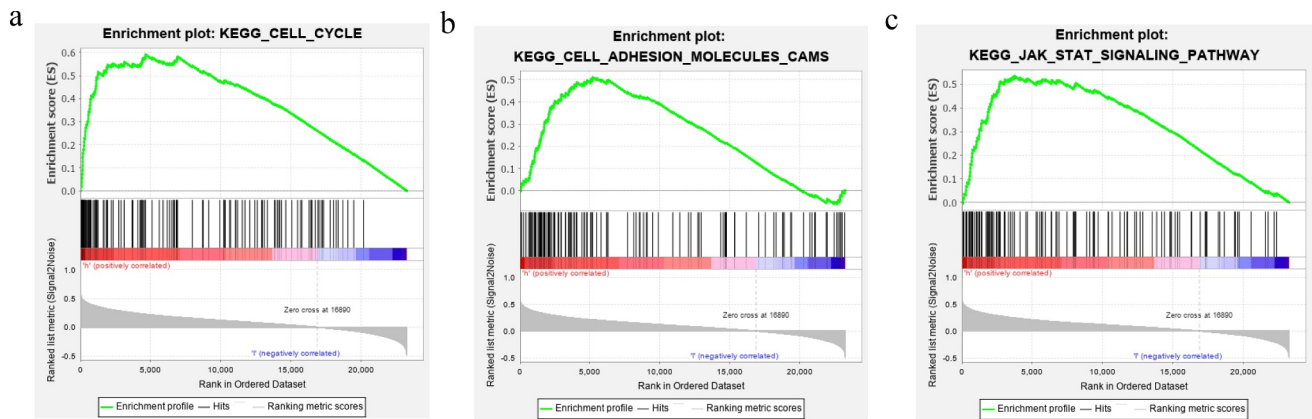
Figure 6. Gene set enrichment analysis (GSEA) of HOXA4 data obtained from the CGGA RNA-seq, CGGA microarray, and TCGA RNA-seq databases. The shared signaling pathways among the three datasets in glioma patients were the (a) Cell cycle, (b) Cell adhesion molecules cams, and (c) Jak stat signaling pathway.

Table 2. The gene set enriches the high HOXA4 expression phenotype based on CGGA-RNA seq data.

Gene set name	NES	NOM p-val	FDR q-value
Cell cycle	1.771875	0.013672	0.102625
Cell adhesion molecules cams	1.7408924	0.0431212	0.107327
Jak stat signaling pathway	1.7032071	0.0384615	0.1192873

NES: normalized enrichment score; NOM: nominal. Gene sets with NOM P-value <0.05 and FDR q-value <0.25 were considered as significantly enriched.

prognosis of glioma patients by enhancing the proliferation, migration, and chemotherapeutic resistance of glioma cells.
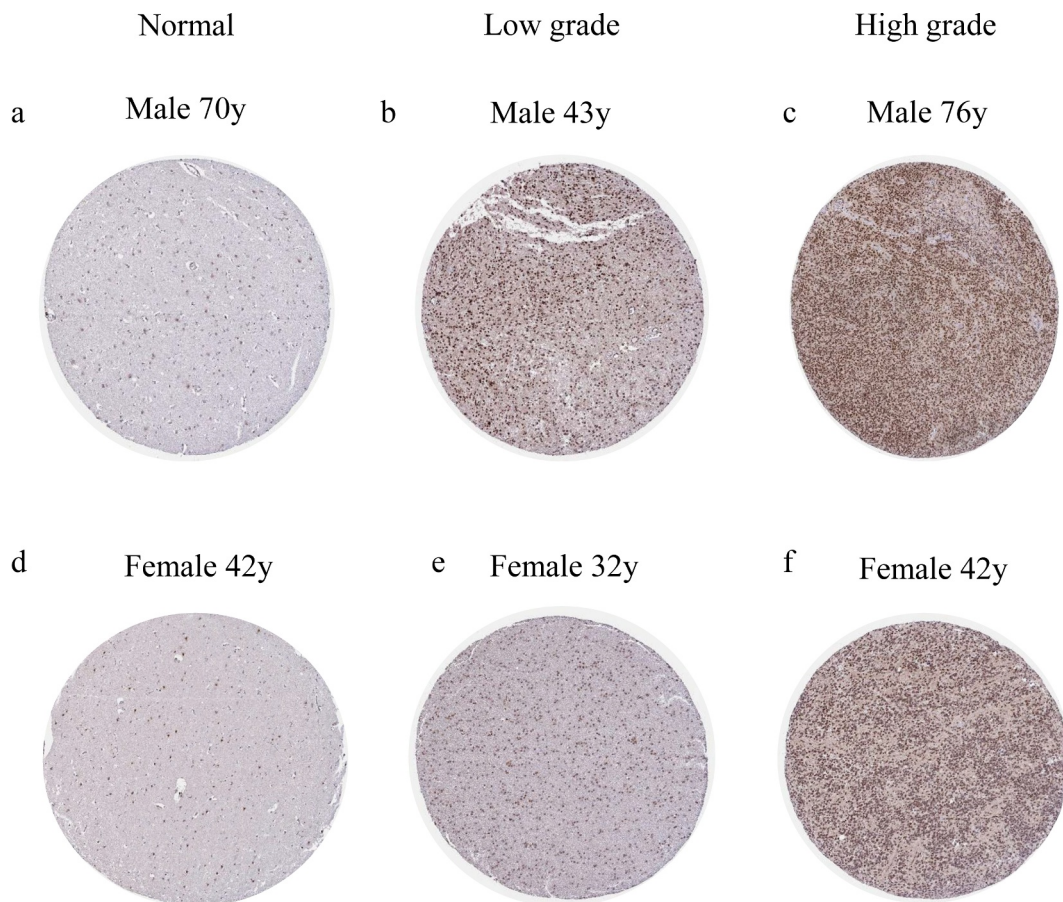
Cell cycle pathway regulated by HOXA4

To answer the question through which mechanism *HOXA4* regulates the malignant characteristics of glioma cells, experiments were performed to verify the role of *HOXA4* on the cell cycle pathway based on the result of GSEA analysis. Flow cytometry results showed that knockdown of *HOXA4* reduced the population of G1 phase cells and increased the population of G2/S phase cells (Figure 8(h,i)). Combined with clone formation experiment, it can be concluded that knockdown of *HOXA4* blocks the cell cycle in the G2/S phase. Moreover, western blotting experiments confirmed that CDK2 and Cyclin E1 expression was increased in the *HOXA4* knockdown group, while CDK4 expression was decreased (Figure 8(j,k)). Combining the above experimental results, we have sufficient evidence to confirm that *HOXA4* can regulate the cell cycle pathway, thus benefiting

malignant progression such as abnormal proliferation of glioma.

Discussion

Gliomas are the most common primary intracranial malignant tumors [28]. Due to their high recurrence rate and unfavorable prognosis, this tumor type is associated with a sizable economic burden [6]. In recent years, although with the development of research, the biological and molecular basis of the occurrence and development of gliomas has been extensively verified, the diagnosis and treatment of tumors have also made significant progress. The prognosis of glioma patients is still poor [29,30]. Therefore, it is necessary further to study the clinical diagnosis and treatment of glioma, explore new risk factors and molecular markers, and develop new therapeutic targets to improve the clinical prognosis of patients with glioma. Previous studies have established that B7-H3 can be used as an immune-checkpoint therapy for glioma patients [31]. As an independent prognostic factor, RGS16 plays a vital role in glioma progression, especially in GBM, and therapeutically targeting RGS16 may have positive effects on the patient response [32]. *HOXA4* is another gene whose potential for diagnosis and prognosis of other cancer types has been established. However, few reports regarding the relationship between *HOXA4* and glioma have been published. In the present study, we aimed to analyze the expression of *HOXA4* in glioma patients and to

	Normal	Low grade	High grade

a Male 70y b Male 43y c Male 76y

d Female 42y e Female 32y f Female 42y

Figure 7. *HOXA4* protein expression in tissues according to the immunohistochemistry analysis of data obtained from the Human Genome Atlas database. Protein expression (a, d) in normal tissue, (b, e) in low-grade glioma tissue, and (c, f) in high-grade glioma tissue.

relate it to diagnosis, prognosis, and clinical outcomes, with the goal of establishing a novel therapeutic target for glioma.

In the present study, the result of survival analysis demonstrated that *HOXA4* overexpression in glioma tissue was associated with poor prognosis in glioma. In previous studies, Ganapathi et al. used RNA sequencing data and qPCR to detect HOX markers for cell division and progression-free survival in patients with primary high-grade serous ovarian cancer (HGSOC). The *HOXA4* risk score can be used as a prognostic biomarker for recurrence in patients with HGSOC in cases where there is no effective biomarker other than mutations in *BRCA1/2* and homologous recombination-related genes [33]. Our results of ROC analysis of the CGGA RNA-seq and CGGA microarray datasets indicated that *HOXA4* also had clinical diagnostic value for WHO II glioma and the results of analyzing all three datasets indicated

clinical diagnostic value for WHO III glioma. These results provided preliminary evidence that *HOXA4* might be a prognostic factor for glioma patients.

To further explore whether *HOXA4* overexpression can be an independent risk factor for the prognosis of glioma, we carried out the univariate and multivariate analyses. According to the univariate and multivariate analysis results, the prognostic value of *HOXA4* expression level was independent of well-known clinical prognostic factors, including age and gender, further attesting to its potential usefulness in clinical practice. This supports previous reports showing that the *HOX* gene family has predictive value for the prognosis of human tumors [34]. MassARRAY and pyrosequencing have been used to measure the methylation of *HOXA3, 7, 9,* and *10* in glioma specimens, with hypermethylation of these genes identified as a prognostic factor for patients with glioma [35]. Hung et al. compared the
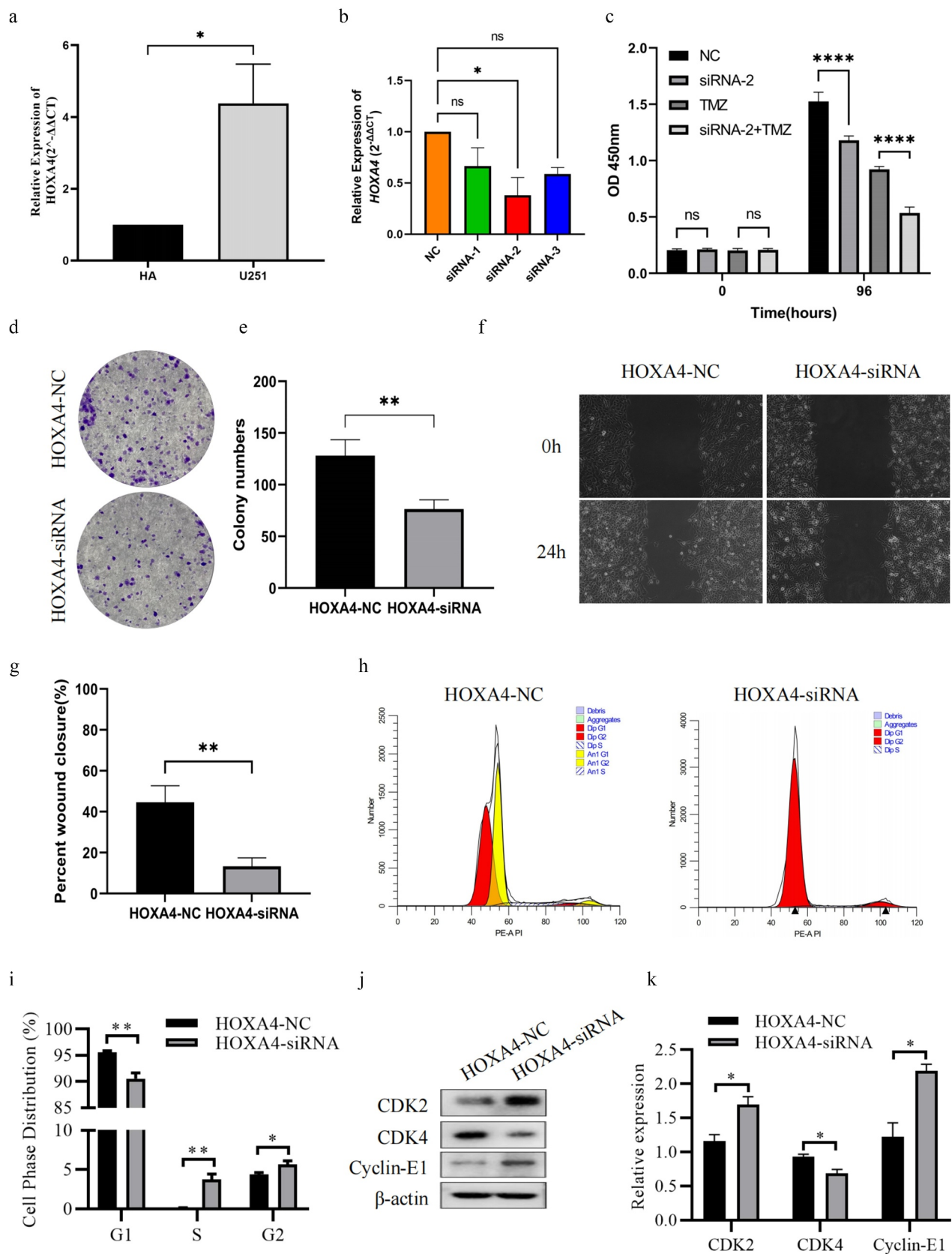
Figure 8. Downregulation of *HOXA4* could inhibit the malignant phenotypes of U251 cells. (a) The expression levels of *HOXA4* in U251 cell lines and HA were determined by RT-qPCR, (b) The interference efficiency of different si-RNA sequences for *HOXA4* in U251 cell line, (c) CCK8 assays in U251 cells with si-RNA2 or NC treated with 40 µM TMZ, (d) Clone formation results of U251 cell lines transfected with si-RNA2 or NC, (e) Analysis of clone formation results, (f) Wound healing assay results U251 cell lines transfected with si-RNA2 or NC, (g) Analysis of wound healing assay results. (h) Flow cytometry results of cell cycle of U251 cell in different groups. (i) The distribution of cell phases in U251 cells of both groups. (j) The expression of cell cycle related proteins in siRNA treated cells. (k) Analysis of protein expressions in western blotting assay. ****$P < 0.0001$, ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$.

expression profiles of pancreatic cancer cells among three genetically engineered mice with different proliferation and metastasis capacities. They found that *HOXA3, B8*, and *C5* overexpression was related to low survival rates for pancreatic cancer, indicating that these genes have an oncogenic role [36]. Furthermore, our in vitro experiments validated these bioinformatics analysis results. The results from our in vitro assays indicated that upregulation of *HOXA4* may promote the proliferation, migration, and chemotherapy resistance of glioma cells, leading to poor prognosis in glioma patients. However, the specific molecular mechanism remains unclear.

Subsequently, to further reveal the specific mechanism of *HOXA4* leading to poor prognosis of patients with glioma, we used GSEA to identify related enriched signaling pathways. GSEA is widely used to reveal the molecular mechanisms underlying diseases and has high reliability. Compared with traditional analysis methods, GSEA has the advantage of using large samples and can avoid the inherent deviation of experimental results caused by artificial threshold settings [37]. The GSEA results indicated that the patients with *HOXA4* overexpression were enriched in tumor-associated signaling pathways such as Cell cycle, Cell adhesion molecules cams, and JAK/STAT signaling pathway. These pathways contribute to the proliferation, migration, and recurrence of cancer cells and are considerably involved in the development and progression of tumors [38–42]. Similar interactions have been described previously. For instance, Previous studies have proven that proteins that regulate cell cycle pathways may lead to the progression, recurrence, and poor prognosis of glioma [43]. Peng C et al. showed that 1,2:5,6-Dihydrogalactitol inhibits the growth of human glioma cells in vivo and in vitro by blocking the cell cycle in G (2)/M phase [44]. Additionally, recent studies have shown that changes in cell adhesion molecules play a key role in tumor progression. They not only define the adhesion function of tumor cells, but also directly affect the classical signal transduction pathways, thereby regulating the metastatic behavior of tumor cells [45]. Moreover, aberrant JAK/STAT signaling has been identified to contribute to cancer progression and metastatic development [46].

Chun Hua Xu et al. revealed that the microRNA-221/222 cluster promotes glioblastoma angiogenesis by activating the cytokine signaling-3 dependent JAK/STAT pathway [47]. Collecting the above results, these above-mentioned cancer-related signaling pathways may be a way for *HOXA4* to lead to a poor prognosis of patients with glioma.

As enlightened by the above-mentioned results, we have conducted a more in-depth validation of the specific mechanism of *HOXA4* function in glioma. First, *in vitro* experiments demonstrated that HOXA4 can promote malignant proliferation of glioma cells by regulating the cell cycle pathway. As reported, regulators of cell cycle pathway can significantly influence the progression of glioma [48]. For example, miR-188, which is targeting β-catenin, damages the proliferation of glioma by inhibiting G1-S transition [49]. Second, *HOXA4*, like other prognostic factors of glioma, can influence the malignant characteristics of glioma cells such as invasion and chemotherapy resistance. As a novel target for the prognosis prediction of glioma, LncRNA GAS5-AS1 can inhibit the invasion of U251 cells through miR-106b-5p/TUSC2 axis [50]. Also, it's reported that the underexpression of *HOXA11* is associated with treatment resistance and poor prognosis in glioblastoma [51]. The above results suggest that *HOXA4* has diverse functions in glioma and may be involved in multiple signaling pathways that mediate malignant progression of glioma. This study further expands the biological functions of *HOXA4* and provides a potentially important biomarker for glioma treatment, in which targeted therapy on *HOXA4* may lead to a better prognosis for glioma patients.

Conclusion

In conclusion, we are the first to identify the *HOXA4* expression associated with the clinical characteristics and prognosis of patients with glioma. At the same time, this study revealed that the overexpression of *HOXA4* could be used as independent prognostics biomarker for glioma, and the downregulation of *HOXA4* can block the cell cycle progression and inhibit glioma cell

proliferation, migration, and chemotherapeutic resistance. It is reasonable to believe that *HOXA4* can be used as a potential diagnostic and therapeutic target to improve the prognosis of glioma.

Data availability statement

All raw data included in this manuscript can be found in the online repository (chengMD2022/HOXA4- (github.com)).

Ethics approval

The study was approved by the Ethics Review Committee of Henan provincial people's hospital (Zhengzhou, China).

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