HOXC6 impacts epithelial-mesenchymal transition and the immune microenvironment through gene transcription in gliomas

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Abstract

Background: Gliomas are the most common primary malignant tumours of the central nervous system (CNS). To improve the prognosis of glioma, it is necessary to identify molecular markers that may be useful for glioma therapy. HOXC6, an important transcription factor, is involved in multiple cancers. However, the role of HOXC6 in gliomas is not clear.

Methods: Bioinformatic and IHC analyses of collected samples (n = 299) were performed to detect HOXC6 expression and the correlation between HOXC6 expression and clinicopathological features of gliomas. We collected clinical information from 177 to 299 patient samples and estimated the prognostic value of HOXC6. Moreover, cell proliferation assays were performed. We performed Gene Ontology (GO) analysis and gene set enrichment analysis (GSEA) based on ChIP-seq and public datasets to explore the biological characteristics of HOXC6 in gliomas. RNA-seq was conducted to verify the relationship between HOXC6 expression levels and epithelial-mesenchymal transition (EMT) biomarkers. Furthermore, the tumour purity, stromal and immune scores were evaluated. The relationship between HOXC6 expression and infiltrating immune cell populations and immune checkpoint proteins was also researched.

Results: HOXC6 was overexpressed and related to the clinicopathological features of gliomas. In addition, knockdown of HOXC6 inhibited the proliferation of glioma cells. Furthermore, increased HOXC6 expression was associated with clinical progression. The biological role of HOXC6 in gliomas was primarily associated with EMT and the immune microenvironment in gliomas. High HOXC6 expression was related to high infiltration by immune cells, a low tumour purity score, a high stromal score, a high immune score and the expression of a variety of immune checkpoint genes, including PD-L1, B7-H3 and CLTA-4.

Conclusions: These results indicated that HOXC6 might be a key factor in promoting tumorigenesis and glioma progression by regulating the EMT signalling pathway and might represent a novel immune therapeutic target in gliomas.

Keywords: HOXC6, Glioma, EMT, Immune, Tumour microenvironment, Biomarker

Introduction

Gliomas are the most common primary malignant tumours of the CNS, accounting for 81% of intracranial malignant tumours in adults and 2% of all malignant tumours in the body. The incidence of glioma is approximately 4.67–5.73/100,000 [1]. As the most lethal glioma, glioblastoma accounts for 70–75% of all diffuse glioma diagnoses, and glioblastoma patients have a median...
overall survival (OS) of 14–17 months [2]. To address this problem and improve the prognosis of gliomas, it is necessary to identify molecular markers that may be useful for glioma therapy.

The homeobox (HOX) gene family, an evolutionarily highly conserved polygenic family, was first found to be involved in developmental regulation in *Drosophila*. In humans, the genes have been reported to be an important family of genes that regulate embryonic development as well as cell growth and differentiation in vivo [3]; these genes have been implicated in numerous tumours. As members of the HOX family, it has been reported that the HOXA7 and HOXA9 genes are vital for establishing and maintaining aberrant HOXA9-HOXA13 gene expression in patients with acute myeloid leukaemia [4]. Many studies have shown that HOXD3 and HOXB13 are upregulated in breast cancer patients [5]. HOXB5, one of the HOXB clusters, is overexpressed in retinoblastoma cell lines and tissues [6].

In recent years, an increasing number of studies have confirmed that HOXC6 is involved in many physiological and pathophysiological processes, such as those in nasopharyngeal carcinoma [7], gastric cancer [8], oesophageal squamous cell carcinoma [9], and prostate cancer [10]. In addition, HOXC6, under the regulation of several signalling pathways, including the TGF-β pathway [11] and Wnt pathway [12], is highly expressed and associated with promoting in tumours [12]. Therefore, HOXC6 represents a potential factor affecting the tumour microenvironment. Immune checkpoint blockade has provided new insights into immunotherapy of gliomas [13]. Nevertheless, because of tumour heterogeneity and immune escape, a detectable clinical benefit from immunotherapy is not achieved in all patients [14, 15]. Thus, new biological targets are urgently needed.

In this study, we downloaded HOXC6 expression data from The Cancer Genome Atlas (TCGA), the Chinese Glioma Genome Atlas (CGGA) and the Rembrandt database and analysed the abnormal expression and prognostic value of HOXC6 in glioma patients based on data from these publicly available databases. Additionally, the correlation of the immune microenvironment was explored using the UCSC XENA and TISIDB databases. Our research focused on HOXC6 as a key factor influencing the EMT-related invasion and migration of gliomas and an immune-related biomarker.

**Methods and materials**

**Patient data collection**

The gene expression and clinicopathological information in TCGA and the Genotype–Tissue Expression (GTEx) database were obtained from the UCSC XENA public repository (https://xenabrowser.net/datapages/), CGGA database (http://www.cgga.org.cn/) and Rembrandt database supplied by gliovis (http://gliovis.bioinfo.cnio.es/).

**Clinical samples and immunohistochemistry (IHC)**

Tissue microarray chips containing samples from a total of 299 patients were obtained from the affiliated hospitals and Shanghai Outdo Biotech Company. The follow-up data for 176 of these 299 patients were used for subsequent survival analysis. All patient information was obtained and used in accordance with the approved protocols of the institutional review boards of the participating institutions. Tissue slides were incubated with rabbit anti-HOXC6 antibody (1:500, Santa Cruz, sc-376330, America), anti-rabbit secondary antibody from Zymed Systems (InvitrogenCA) and 3,3'-diaminobenzidine to visualize IHC labelling. The slides were lightly counterstained with crystal violet. Normal rabbit IgG was used to verify the specificity of the IHC labelling. The results were analysed by ImageProPlus software (version 6.0). We determined the positive expression intensity of IHC by employing the mean optical density [16], which indicates the average reaction intensity of all selected objects in a field of vision. The mean optical density was calculated using the IOD Sum/Area Sum method. To ensure the authenticity of the measurement results, we adjusted the optical density of all the images and set identical parameters in HIS mode prior to measurement.

**Pan-cancer analysis of HOXC6 mRNA expression**

HOXC6 expression data across different cancers compared with normal tissues were downloaded from Oncomine (www.oncomine.org) based on a [fold change]>1.5, p value < 0.001 and top 10% gene ratio. Table 1 provides information about the datasets that met these thresholds. Expression data of 33 types of cancer vs. normal tissue were downloaded from the TCGA and GTEx databases. Univariate hazard ratios for each cancer were also analysed and presented with forest plots.

**Correlation with clinicopathological features and prognosis analysis**

The relationships between HOXC6 mRNA expression and different clinicopathological features were analysed based on TCGA, CGGA, and Rembrandt data and clinical samples. Kaplan–Meier analysis and multiple Cox regression analysis of the prognostic effect of HOXC6 mRNA expression were performed by using the survival and survminer R packages; correspondingly, the receiver operating characteristic (ROC) curve was analysed by using the timeROC R package. The nomogram and calibration plots were constructed using the RMS package of R software.
Bioinformatics analysis
We screened HOXC6 positively correlated genes (Spearman’s coefficient > 0.3, p < 0.05) from glioma data in public databases and a list of HOXC6 target genes from ChIP-seq. Based on their intersection, GO analyses were employed by using the R package clusterProfiler [17]. GSEA was performed on the basis of HOXC6 expression in TCGA samples [18]. Metagenes of immune infiltrating cells were downloaded from TISIDB (http://cis.hku.hk/TISIDB/index.php), and single-sample gene set enrichment analysis (ssGSEA) was conducted via the GSVA R package. All association analyses were investigated using Spearman correlation analysis. Stromal, purity and immune scores were available in the UCSC XENA public repository. The packages ggplot2, ggradar, UpSetR, and ComplexHeatmap in R software were used for data visualization. The analysis employed a list of immune checkpoint proteins gathered from an important previous study [19].

Cell culture and transfection
U251, T98G, U118 and U87 human glioma cells and human astrocytes (HA) were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Gibco, C1199500BT, Canada) with 10% foetal bovine serum (FBS, Gibco, 10,091,148, Canada) and supplemented with 1x penicillin/streptomycin (Gibco, 15140-122, Canada). All cultures were maintained in a 37 °C, 5% CO2 incubator (TFS3111, America).

The overexpression lentiviral vector for HOXC6 was constructed by Genechem (GSDL0168387, China). The sequences were cloned into the lentiviral vector GV358 with a Flag-tag. A lentiviral HOXC6 short hairpin RNA (shRNA) was purchased from Genechem (GIEL0177253, China). The shRNA sequence targeting human HOXC6 complementary DNA was 5’-GAC CAGAAAAGCAGTATCCAG-3’. A scrambled shRNA was included as a negative control (NC). The target sequence was inserted into the GV248 lentiviral vector (Genechem, China).

Western blot analysis
In this study, RIPA buffer (Cell Signaling Technology, 9806, America) was used to extract proteins from cells or tissues. Approximately 25 µg of the protein per lane was detected according to a standard protocol of sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. The membranes were incubated overnight at 4 °C with HOXC6 (1:500, Abcam, ab151575, Britain) antibody. GAPDH (1:10000, Proteintech, 60004-1-lg, China) was used as a protein loading control.

RT q-PCR
Total RNA was extracted from cells using TRIzol Reagent (Sigma–Aldrich, T9424, America) in a ventilator according to the manufacturer’s guidelines. RNA was reverse-transcribed into cDNA using the PrimeScript RT Reagent Kit (Takara, RR047, Japan). Quantitative real-time PCR was performed using SYBR Premix Ex Taq™ II (Takara, RR047, Japan) in an ABI StepOnePlus system. The primers were provided by Sheng Kong Company, Shanghai, China. The sequences of the primers were as follows: HOXC6, CGCACAACCTCTTCTTCCACC and TCACCTGGAGGGCAATCT; MMP9, CAGTACCGGAGAAAGGCCATT and CAGGATGTGATGCTACGTAG; CDH1, AGTCACCTGACACCAAGTAT and ATCGTGTGCTCTAGGATTTGT; CDH2, CGTAAAGGATCAACCCCATACA and TTCAAGTCGATGCTTGGACC; DKK1, TACCAGACCACTGAGAAT and TACCCTCTTTCAGGATTTTGG; IDO, CTGCCTGAGATCGTGGC and CTGGGAGATCAGATTTGAG. The expression of the target genes was normalized to GAPDH, which served as an internal control.

Table 1: The Significant Changes in HOXC6 Expression at the Transcription Level between Different Types of Brain and CNS Cancers vs. Normal Brain Tissues (Oncomine Database)

| Type of brain and CNS cancer versus normal brain tissues | Fold Change | P Value | T Test | Reference |
|--------------------------------------------------------|-------------|---------|--------|-----------|
| Glioblastoma                                           | 2.448       | 2.26E-13| 8.604  | Murat Brain Statistics |
| Anaplastic Oligoastrocytoma                            | 1.73        | 7.70E-04| 5.609  | Bredel Brain 2 Statistics |
| Glioblastoma                                           | 1.533       | 1.43E-08| 8.502  | Bredel Brain 2 Statistics |
| Glioblastoma                                           | 1.939       | 5.54E-08| 7.565  | Lee Brain Statistics |
| Glioblastoma                                           | 1.972       | 1.24E-16| 9.853  | Sun Brain Statistics |
| Anaplastic Astrocytoma                                 | 1.691       | 8.17E-05| 4.527  | Sun Brain Statistics |
| Brain Glioblastoma                                     | 2.714       | 6.23E-09| 14.195 | TCGA Brain Statistics |
control. The comparative CT (ΔΔCT) method was used to analyse fold changes.

**Cell proliferation assay**

**Cell counting Kit-8 assay**

To measure cell proliferation, we used Cell Counting Kit-8 (CCK-8) (Dojindo, CK18, Japan). Both control and transfected cells were seeded into 96-well plates (Corning, 3599, America) at an initial density of 1000 cells/well. Then, CCK8 reagent (10 µl/well) was added to each well. Twenty-four hours after seeding, cell viability was assessed with a microplate reader (Thermo, Multiskan-Spectrum, America) using CCK8 reagent as described above. This was considered day 0. Later, cell viability was measured every 24 h. Cell growth was indicated by the fold change from day 0 to day 4 and was graphed.

**Chromatin immunoprecipitation (ChIP) and sequencing**

We transfected U251 cells with the HOXC6 overexpression lentiviral vector with a flag tag. Then, ChIP was performed using the SimpleChIP Enzymatic Chromatin IP Kit (Cell Signaling Technology, 9003, America) according to the manufacturer’s instructions. The antibody DYKD-DDDK (Cell Signaling Technology, 14,793 S, America) was used to pull down HOXC6 in the positive control and NC. Purified ChIP-DNA was verified on an agarose gel to ensure proper fragmentation and then sent to the company for ChIP-seq. After that, we used FastQC and MultiQc software for follow-up data quality control. Bowtie2 software was used for genome sequence alignment, and Picard was used to remove duplicate reads. MACS2 software was used for peak calling analysis, and the R package cheappeakAnno and MEME software were used for DNA binding motif enrichment analysis for annotation and statistical assessment of the analysis results [20]. Then, we performed integrative analysis on HOXC6 differentially regulated genes and ChIPseq annotated targets [21] (Additional file 1).

**Results**

**Overexpression of HOXC6 in Gliomas**

Differences in HOXC6 expression between tumour and normal tissues from patients with different cancers were detected by using data from the Oncomine database. HOXC6 mRNA was overexpressed in many cancers, especially in brain and CNS cancer, lung cancer, lymphoma and prostate cancer. In addition, the expression of HOXC6 in ovarian cancer and melanoma was low (Fig. 1A). According to the TCGA and GTEx databases, HOXC6 expression was further analysed and found to be upregulated in most types of tumours but reduced in kidney chromophobe (KICH) and uterine corpus endometrial carcinoma (UCEC) (Fig. 1B). As shown in Fig. 1C, HOXC6 was considered a risk factor for glioblastoma multiforme (GBM), brain lower grade glioma (LGG), adrenal cortical carcinoma (ACC), colon adenocarcinoma (COAD), head and neck squamous cell carcinoma (HNSC), liver hepatocellular carcinoma (LIHC) and lung adenocarcinoma (LUAD) with p-values of less than 0.05. Furthermore, we analysed the significant changes in HOXC6 expression at the transcriptional level between different forms of brain and CNS cancers vs. normal brain tissues using the Oncomine database (Table 1). Generally, HOXC6 plays a significant role in brain and CNS cancers. The abbreviations for each cancer are summarized in Additional file 2: Table S1.

Considering the emerging role of epigenetic regulators as targets for cancer therapy, we tested whether HOXC6 was differentially expressed in the context of gliomas. For this, we analysed the protein expression level of HOXC6 in glioma tissues in 299 patient samples using IHC, which verified that the protein expression of HOXC6 was positively correlated with a high grade of glioma (Fig. 2A, B). HOXC6 expression correlated positively with glioma grade in the TCGA dataset (Fig. 2C). Consistent with the TCGA database, the HOXC6 expression data in the CGGA and Rembrandt databases showed the same tendency (Fig. 2D and E). Here, we subdivided the TCGA samples into gliomas with a wild-type isocitrate
dehydrogenase (IDH) gene, with a mutant-type IDH gene, with 1p/19q codeletion, and without 1p/19q codeletion. According to the TCGA database, gliomas with a wild-type IDH gene and gliomas without 1p/19q codeletion had a high level of HOXC6 expression consistent with the group of gliomas with a mutant-type IDH gene and with 1p/19q codeletion (Fig. 2F, H). Consistent with the TCGA database, the expression of HOXC6 in CGGA showed a consistent tendency (Fig. 2G and I).

Correlation between HOXC6 expression and prognosis in patients with gliomas

The relationship between HOXC6 expression and prognosis in patients with gliomas was further analysed using our clinical samples, which illustrated that the high expression of HOXC6 was related to a short OS (Fig. 3A). Additionally, we found that low HOXC6 expression was associated with a longer OS in the TCGA database (Fig. 3B), which was consistent with the CGGA and Rembrandt databases (Fig. 3C, D).

Then, we structured the ROC curve on account of the clinical samples, TCGA, CGGA and Rembrandt datasets to explore the value of HOXC6 for glioma diagnosis according to the area under the ROC curve (AUC). All the above ROC curves verified the high diagnostic value of HOXC6 for the survival prognosis of glioma (Fig. 3E–H).

The prognostic nomogram with a risk classification system for 2-, 3- and 5-year survival rates of glioma based on TCGA was performed (n = 703, Fig. 3I). To test the efficiency of the new nomogram, 200 bootstrap re-samplings were performed for internal verification through the calibration chart in the two independent cohorts of TCGA and CGGA, which indicated a good calibration effect of the nomogram (Fig. 3J, K). Univariate (HR = 5.678, p < 0.001) and multivariate (HR = 1.649, p = 0.022) Cox regression analyses were then conducted, and factors related to the prognosis of glioma were chosen (Table 2). Baseline clinical data in the TCGA, CGGA datasets and clinical patients can be found in Additional file 5: Table S4, Additional file 6: Table S5, Additional file 7: Table S6.

Subsequently, we conducted verification tests to verify that HOXC6 upregulation is strongly associated with...
poor prognosis in patients with gliomas. Western blot and RT qPCR showed varied expression levels of GATA3 in U118, T98G, U251, U87 and HA cells (Fig. 4A, B). After transfection in U251 and U87 cells, HOXC6 expression was drastically decreased (Fig. 4C, D). Cell proliferation assays, including the CCK-8 assay (Fig. 4E, F), colony-forming assay (Fig. 4G, H) and EdU assay (Fig. 4I, J), were performed using the transfected cells. All these assays revealed that HOXC6 notably promoted cell proliferation in glioma cells, which demonstrated the correlation between low HOXC6 expression and good prognosis in gliomas.

**HOXC6-related EMT-associated tumour invasion and metastasis in gliomas**

According to the aforementioned results, HOXC6 may have an important impact on the biological functions...
of gliomas. Since HOXC6 is a significant transcription factor in many tumours, ChIP-seq was performed. The HOXC6-bound DNA motifs were identified by using MEME software (Fig. 5A), and the distribution of HOXC6 peak binding locations across the genome is shown in Fig. 5B. To further clarify the biological roles of HOXC6 expression in glioma, we selected 5225 genes from the TCGA dataset (Spearman’s R \(>0.3, p<0.05\)), 4064 genes from the CGGA dataset (Spearman’s R \(>0.3, p<0.05\)) and 3330 target genes from ChIP-seq (Fig. 5C). Then, the intersection of genes selected in the three datasets was explored by GO analysis. We found that the most involved terms were EMT, positive regulation of cell adhesion, extracellular matrix organization, positive regulation of EMT, T-cell activation, T-cell differentiation and so on when the gene functions were sorted by p-adjust \(<0.05\) and FDR \(<0.2\) (Fig. 5D). The network diagram of the enrichment map showed that the enriched terms were centrally concentrated in EMT-related pathways as well as the immune response (Fig. 5E).

After GSEA, it was easy to find that EMT-related pathways and inflammatory responses were positively affected by HOXC6 using the ridge plot (Fig. 6A). Then, we used a Venn diagram to indicate the intersection of HOXC6 differentially regulated genes and ChIP-seq annotated targets (Fig. 6B). We listed the ChIP-seq annotated targets differentially expressed in the sequencing results at the thresholds | fold change | \(>2\) and p

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**Fig. 3** HOXC6 predicts a poor prognosis in glioma patients. **A–D** Higher HOXC6 expression portended shorter OS in patients with gliomas based on clinical samples (\(n=176\)) and the TCGA (\(n=695\)), CGGA (\(n=656\)) and Rembrandt datasets (\(n=397\)). **E–H** ROC curves based on the above samples. I Nomogram for predicting 2-, 3- and 5-year survival in glioma patients based on the TCGA dataset. **J–K** Calibration curves were used to predict the 2-, 3-, and 5-year survival in the TCGA and CGGA datasets.
value < 0.05 (Fig. 6C). Sequencing data on EMT-related genes and ChIP-seq annotated targets can be found in Additional file 8: Table S7. In addition, circular heatmap based on the RNA-seq, TCGA, CGGA and Rembrandt databases demonstrated that HOXC6 expression levels had a positive relationship with most EMT-related biomarkers (Fig. 6D).

**HOXC6 is involved in the regulation of the Tumour Immune Microenvironment**

Since previous experimental data showed a relationship between HOXC6 expression and glioma biological progression as well as the immune response, we further considered whether the expression of HOXC6 was relevant to the formation of the tumour immune microenvironment. To test this hypothesis, we first analysed the relationship between the HOXC6 expression level and tumour purity, which showed a negative association (Fig. 7A). Then, the scatter diagram was displayed to show the positive association between HOXC6 expression and the stromal score (Fig. 7B). In addition, we investigated the relationship between HOXC6 expression and the immune score, which remained positive (Fig. 7C). The above results demonstrate that HOXC6 expression has a close relationship with the tumour immune microenvironment.

Hence, we next used immune cell GSVA to assess whether certain immune infiltrates might be associated with elevated HOXC6 expression in TCGA and CGGA. A heatmap was used to show the relationship between the expression level of HOXC6 and 28 infiltrating immune cell features based on the TCGA and CGGA databases (Fig. 7D). Most immune infiltration levels in the HOXC6 high-expression group were higher than those in the HOXC6 low-expression group. The lollipop diagram showed the ordering of HOXC6 in relation to these immune cell infiltrates (Fig. 7E). Detailed correlations of HOXC6 expression levels with the mentioned infiltrating immune cell features are shown in Additional file 3: Table S2.

**Table 2** Univariate and multivariate analyses of overall survival based on TCGA data

| Characteristic            | Total (N) | Univariate analysis |          |          |
|---------------------------|-----------|---------------------|----------|----------|
|                           |           | Hazard ratio (95% CI) | P value  | Hazard ratio (95% CI) | P value  |
| Sex                       | 695       |                      |          |          |
| Female                    | 297       | Reference            |          |          |
| Male                      | 398       | 1.262 (0.988–1.610)  | 0.062    | 1.253 (0.951–1.652)  | 0.109    |
| Age                       | 695       |                      |          |          |
| <=60                      | 552       | Reference            |          |          |
| >60                       | 143       | 4.668 (3.598–6.056)  | <0.001   | 1.502 (1.104–2.043)  | 0.010    |
| 1p/19q codeletion          | 688       |                      |          |          |
| Codel                     | 170       | Reference            |          |          |
| Non-codel                 | 518       | 4.428 (2.885–6.799)  | <0.001   | 1.342 (0.806–2.234)  | 0.258    |
| IDH status                | 685       |                      |          |          |
| WT                        | 246       | Reference            |          |          |
| Mut                       | 439       | 0.117 (0.090–0.152)  | <0.001   | 0.350 (0.228–0.536)  | <0.001   |
| WHO grade                 | 634       |                      |          |          |
| G2                        | 223       | Reference            |          |          |
| G3                        | 243       | 2.999 (2.007–4.480)  | <0.001   | 1.824 (1.184–2.810)  | 0.006    |
| G4                        | 168       | 18.615 (12.460–27.812)| <0.001 | 4.200 (2.474–7.130)  | <0.001   |
| HOXC6                     | 695       |                      |          |          |
| Low                       | 347       | Reference            |          |          |
| High                      | 348       | 5.678 (4.243–7.598)  | <0.001   | 1.649 (1.076–2.529)  | 0.022    |
expression levels with the mentioned immune checkpoints are shown in Additional file 4: Table S3.

To identify the positive relationship between HOXC6 expression and EMT-related genes, we performed RT q-PCR assays (Fig. 8A). The results confirmed the positive association between HOXC6 expression and the EMT-related genes we analysed above. Then, we conducted RT q-PCR assays to verify the positive correlation between the expression of HOXC6 and the abovementioned immune checkpoint proteins (Fig. 8B). As we expected, a positive significant association between HOXC6 and key immune checkpoint proteins was shown.

Discussion

Despite great progress in glioma surgery, radiotherapy [22] and chemotherapy [23], the prognosis of glioma patients is still poor. Genetic examination can be used to guide radiotherapy and chemotherapy for glioma. For instance, patients with mutations in IDH1 and IDH2 have a better prognosis and clinical response after radiotherapy and chemotherapy [24, 25]. In addition, people with 1p19q non-codeletion were considered insensitive to radiotherapy [26, 27]. Consequently, there is an urgent need for more therapeutic targets and molecular markers.

HOXC6 is a member of the HOXC cluster, located on chromosome 12Q13.13. The protein encoded by HOXC6 consists of 235 amino acids, of which amino acids 145–198 constitute the classical homeodomain that mediates HOXC6 binding to the target gene promoter region. However, the function of regions 1-144 and 199–235 is unclear [28]. Therefore, we used ChIP-Seq to detect its target genes to analyse its biological function. Herein, we demonstrated that HOXC6 was overexpressed in many cancers, especially in glioma. Moreover, we found that HOXC6 was significantly upregulated in high-grade glioma, a wild-type IDH gene and 1p19q non-codeletion, and these

![Fig. 4](image-url)
characteristics indicate tumour malignancy and are insensitive to chemotherapy [29].

In a previous study, high HOXC6 expression resulted in poor survival in patients with multiple cancers [7, 30]. As expected, our study showed that patients with high levels of HOXC6 tended to have a shorter OS than those with low levels in clinical samples and the TCGA, CGGA and Rembrandt datasets. By assessing individualized prognostic forecasts in the nomogram [31], we confirmed HOXC6 as a prognostic biomarker of glioma. The calibration plots of the two datasets were highly fitted, illustrating that the nomogram performed well in predicting 2-, 3- and 5-year survival in patients with gliomas. Therefore, we inferred that HOXC6 can act as a predictor for the clinical prognosis of glioma patients.

To further explore the biological function of HOXC6 in glioma, GO analyses were conducted to reveal that HOXC6 was strongly associated with the functions of tumour progression, apoptosis, EMT and inflammation. GSEA further revealed significant enrichment of EMT,
angiogenesis, inflammatory response, and the IFN-α pathway, IFN-γ pathway and IL6/JAK/STAT3 pathway. The functional network binding to hub genes indicated that the EMT signalling pathway may be the core pathway by which HOXC6 regulates tumour progression.

EMT is an important process in which epithelial cells lose cell polarity and adhesion and become mesenchymal cells. This is closely related to the existence of glioma stem cells discovered in recent years and ultimately leads to a high capacity for migration and invasion, resistance to radiotherapy and chemotherapy and tumour recurrence [32]. As reported in previous studies of other types of cancer, HOXC6 exhibited the biological function of matrix remodelling, cell migration, invasion and metastasis in other types of malignancies, such as laryngeal cancer [33], cervical carcinoma [34] and lung cancer [35]. To further validate the role of HOXC6 in the glioma EMT process, a series of representative genes of the EMT process were selected and analysed to show their high correlation with HOXC6. In previous studies, the interaction of HOXC6 and EMT biomarkers, including MMP9 [36], vimentin [37] and TWIST1 [38], has also been confirmed. In our study, we verified that the expression of MMP9 and CDH2 were decreased, while the expression of CDH1 and DKK1 were increased in HOXC6 knockdown cells. In addition, RNA sequencing further reinforced that knocking down HOXC6 in vitro resulted in low expression of EMT-related genes.

In addition, a potential association of EMT with tumour immune environment formation has been shown in recent studies. On the one hand, the cytokines generated upon EMT increase tumour infiltration by immune cells [39]. On the other hand, the EMT process may weaken the actions of immune effector cells via regulator T cells (Tregs) and interferon gamma signals, leading to an escape from recognition and killing by the immune system [40]. These findings may partly account for the results of our study: HOXC6 expression is positively correlated with the stromal and immune scores of glioma patients but negatively correlated with tumour purity. Thus, our findings might illustrate the potential dual value of HOXC6 both in EMT targeting and in immunological therapy.
Moreover, gene set variation analysis indicated that there was a close link between HOXC6 and infiltrating immune cells, including immunosuppressive components such as Tregs and myeloid-derived suppressor cells (MDSCs). Several studies have demonstrated that the proportion of Tregs increases with the degree of glioma malignancy [41]. Tregs suppress T-cell proliferation by capturing IL2 and regulate the function of effector T cells by secreting inhibitory cytokines, including IL-10 [42]. More importantly, CTLA-4 on Treg cells downregulates the expression of CD80 and CD86 on DCs, thereby inducing DC tolerance, which further inhibits the ability of T cells to express IDO [43]. In this case, cytokines produced by MDSCs and Tregs form a positive feedback loop to enhance immunosuppression. The TCGA and CGGA data analysis showed that HOXC6 is strongly
linked to CTLA-4, IDO, and TDO. Therefore, HOXC6 is very likely to promote the functions of these tumour-infiltrating immunosuppressive cells.

In recent decades, checkpoint inhibitors have achieved remarkable results in tumour treatment and have become the most advanced immunotherapy in clinical application [44]. As the most-studied checkpoint, PD-L1 involves a complex mechanism, and its important relationship with interferon has been revealed in previous studies. PD-L1 is widely expressed in glioma, participates in tumour-induced immunoregulation of infiltrating T cells and plays the role of a negative prognostic factor [45]. As important cytokines secreted by T cells, interferons have a dual role in the tumour process. Interferons can activate DCs to release tumour necrosis factor-related apoptosis-inducing ligands, thereby strengthening the functions of tumour-infiltrating natural killing cells and cytotoxic T lymphocytes (CTLs) [46]. Nevertheless, in some cases, interferons upregulate the expression of PD-L1 on the tumour cell surface and enhance the infiltration of immunosuppressive cells, leading to the inhibition of the anti-tumor immune response and the occurrence of tumour immune escape [47]. In our study, the Spearman correlation coefficients between HOXC6 and PD-L1 were 0.44 in TCGA gliomas and 0.55 in CGGA gliomas. GSEA showed a clear enrichment of interferon signalling pathways. These results indicate that HOXC6 expression in glioma patients may predict the therapeutic efficacy of PD-L1 blocker therapy.

In addition, using the TCGA and CGGA datasets, we demonstrated that B7-H3 showed the strongest correlation with HOXC6 among the members of the B7 ligand family. B7-H3 is associated with poor prognosis and is a valuable guide for immunotherapy. In malignant tissues, B7-H3 inhibits tumour antigen-specific immune responses, leading to tumorigenic effects [48]. B7-H3 also has non-immuno-pro-tumour effects, such as in angiogenesis, drug resistance, EMTEMT and influencing tumour cell metabolism [49]. In our study, the Spearman correlation coefficients between HOXC6 and B7-H3 were 0.64 in TCGA gliomas and 0.55 in CGGA gliomas. HOXC6 exhibited widespread correlations with other immune checkpoints, such as LGALS9 and CD96. PD-L1 and B7-H3 expression levels were decreased in HOXC6 knockdown glioma cell lines. These results illustrate the extremely broad and promising applications of HOXC6 for immune checkpoint treatments.

Of course, there are some limitations in our research. First, although our results are based on a large number of bioinformatics analyses, RNA-seq and ChIP-seq, the specific in vivo mechanisms of how HOXC6 regulates immunity and EMT still need to be further explored. Second, we also need more animal models to validate our findings and advance the clinical application of HOXC6 as a glioma immunotherapy target.

**Conclusions**

In conclusion, HOXC6 is a prognostic indicator in glioma. Moreover, our study broadens our understanding of the functions of HOXC6 transcription target genes. HOXC6, which is associated with EMT and the immune microenvironment, is expected to be a potential therapeutic target for glioma. These findings may lead to new perspectives on the treatment of
gliomas. We provide a flow chart to better reflect the design of our bioinformatics investigation in Additional file 1: Figure S1.

Supplementary information
The online version contains supplementary material available at https://doi.org/10.1186/s12935-022-02589-9.

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Author contributions
HJT, ZH and JJ conducted the bioinformatics analysis and drafted the original manuscript. JH helped collect the databases and conducted the IHC and proliferation assays. WJ, JH, BX and ZB were involved in data acquisition. Junfei S and Jun S revised the manuscript and gave administrative, technical or material support. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets in our study are available in the UCSC XENA public repository (https://xenabrowser.net/datasetpages/), CGGA database (http://www.cgga.org.cn/) and Rembrandt database supplied by gliovis (http://gliovis.bioinfo.cnio.es/).

Declarations
Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Conflict of interest
The authors declare that they have no competing interests.

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References
1. Q.T. Ostrom, L. Bauchet, F.G. Davis, I. Deltour, J.L. Fisher, C.E. Langer, M. Pekmezci, J.A. Schwartzbaum, M.C. Turner, K.M. Walsh, M.R. Wenisch, J.S. Barnholtz-Sloan, The epidemiology of glioma in adults: a state of the science review, Neuro Oncol 16 (2014) 896–913. https://doi.org/10.1093/neuonc/nou087.
2. A.M.H. Chen, S. Callier, T. Franz, N. Fox, P. Thornton, M. Norfolk, Development and validation of the self-care counseling rubric (SCCR) to assess student self-care counseling skills, Curr Pharm Teach Learn 11 (2019) 774–781. https://doi.org/10.1016/j.cptl.2019.04.006.
3. C. Xu, B. Li, S. Zhao, B. Jin, R. Ji, G. He, H. Xu, MicrORNA-186-5p Inhibits Proliferation And Metastasis Of Esophageal Cancer By Mediating HOX9, Onco Targets Ther 12 (2019) 8905–8914. https://doi.org/10.2147/OTT.327720.
4. H. Luo, F. Wang, J. Zha, H. Li, B. Yan, Q. Diu, F. Yang, A. Sobh, C. Vulpe, L. Drusbolsky, C. Cogle, J. Chepelev, B. Xu, S.D. Nimer, J. Licht, Y. Qiu, B. Chen, M. Xu, S. Huang, CTCF boundary remodels chromatin domain and drives aberrant HOX gene transcription in acute myeloid leukemia, Blood 132 (2018) 837–848. https://doi.org/10.1182/blood-2017-1184319.
5. H. Hut, J.Y. Lee, H.J. Yun, B.W. Park, M.H. Kim, Analysis of HOX gene expression patterns in human breast cancer, Mol Biotechnol 56 (2014) 64–71. https://doi.org/10.1007/s12033-013-9682-4.
6. H. Xu, H. Zhao, J. Yu, HOX85 promotes retinoblastoma cell migration and invasion via ERK1/2 pathway-mediated MPPs production, Am J Transl Res 10 (2018) 1703–1712.
7. S.L. Chang, T.C. Chan, T.J. Chen, S.W. Lee, L.C. Lin, K.T. Win, HOXC6 Over-expression Is Associated With Ki-67 Expression and Poor Survival in NPC Patients, J Cancer 8 (2016) 1647–1654. https://doi.org/10.1016/j.jca.18893.
8. S.W. Chen, Q. Zhang, Z.F. Xu, H.P. Wang, Y. Shi, F. Xu, W.J. Zhang, P. Wang, Y.L. HOXC6 promotes gastric cancer cell invasion by upregulating the expression of MMP9, Mol Med Rep 14 (2016) 3261–3268. https://doi.org/10.3892/mmr.2016.3640.
9. L.Y. Shen, M.Y. Fan, B. Dong, W.P. Yan, K.N. Chen, Increased HOXC6 expression predicts chemotherapy sensitivity in patients with esophageal squamous cell carcinoma, Oncol Lett 14 (2017) 4835–4840. https://doi.org/10.3892/ol.2017.6772.
10. Z. Luo, P.J. Farnham, Genome-wide analysis of HOX4 and HOX6 regulated genes and binding sites in prostate cancer cells, PLoS One 15 (2020) e0228590. https://doi.org/10.1371/journal.pone.0228590.
11. B. Bath, J. Lavison, D. Ma, C. Trask, Self-reported use of family physician, chiropractor and physiotherapy services among adult Canadians with chronic back disorders: an observational study, BMC Health Serv Res 18 (2018) 970. https://doi.org/10.1186/s12913-018-3790-6.
12. L. Qi, J. Chen, B. Zhou, K. Xu, K. Wang, Z. Fang, Y. Shao, Y. Yuan, S. Zheng, W. Hu, HomeoboxC6 promotes metastasis by orchestrating the DKK1/Wnt/beta-catenin axis in right-sided colon cancer, Cell Death Dis 12 (2021) 537. https://doi.org/10.1038/s41419-021-03630-x.
13. S. Goswami, T. Walle, A.E. Cornish, S. Basu, S. Anandhan, I. Fernandez, L. Vence, J. Blando, H. Zhao, S.S. Yadav, M. Ott, L.Y. Kong, A.B. Heimberger, J. de Groot, B. Sepehi, M. Overman, S. Kopetz, J.P. Allison, D. Péter, P. Sharma, Immune profiling of human tumors identifies CD73 as a combinatorial target in gliblastoma, Nat Med 26 (2020) 39–46. https://doi.org/10.1038/s41591-019-0699-y.
14. Q.T. Ostrom, H. Gittleman, J. Xu, C. Kruchko, J.S. Barnholtz-Sloan, CBTRUS Statistical Report: Primary Brain and Other Central Nervous System Tumors Diagnosed in the United States in 2009–2013, Neuro Oncol 18 (2016) v1–v75. https://doi.org/10.1093/neuonc/nov207.
15. A. Woehrey, L. Bauchet, J.S. Barnholtz-Sloan, Glioblastoma survival: has it improved? Evidence from population-based studies, Curr Opin Neurol 27 (2014) 666–674. https://doi.org/10.1097/WCO.0000000000000144.
16. Y. Horai, T. Kakimoto, K. Takemoto, M. Tanaka, Quantitative analysis of histopathological findings using image processing software, J Toxicol Pathol 24 (2014) 351–358. https://doi.org/10.1293/toxpathol.2014-0031.
17. G. Yu, L.G. Wang, Y. Han, Q.Y. He, clusterProfiler: an R package for comparing biological themes among gene clusters, OMICS 16 (2012) 284–287. https://doi.org/10.1089/omi.2011.0118.
18. A. Subramanian, P. Tamayo, V.K. Mootha, S. Mukherjee, B.L. Ebert, M.A. Gillette, A. Paulovich, S.L. Pomeroy, T.R. Golub, E.S. Lander, J.P. Mesirov, Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles, Proc Natl Acad Sci U S A 105 (2008) 15445–15450. https://doi.org/10.1073/pnas.0506580102.
19. Sharger SH, Kiel C. SnapShot: APC/T cell immune checkpoints. Cell. 2020;183(4):1142e1141. https://doi.org/10.1016/j.cell.2020.10.007.
20. Liu P, Jiang W, Zhou S, Gao J, Zhang H. Combined analysis of ChIP sequencing and gene expression dataset in breast cancer. Pathol Oncol Res. 2017;23:361–8. https://doi.org/10.1007/s12253-016-0116-z.

21. Tripathi SK, Chen Z, Laijo A, Kundur K, Nousainen K, Aoji T, Ricano P, Ponce I, Hrdlickova B, Tuomela S, Lajajala E, Sala V, Kumar V, Wijemenga C, Lahdesmaki H, Lahesmaa R. Genome-wide analysis of STAT3-mediated transcription during early human Th1 cell differentiation. Cell Rep. 2017;19:1888–901. https://doi.org/10.1016/j.celrep.2017.05.013.

22. E. Kokavvakij, K.J. Anderson, F.S. Varn, K.C. Johnson, S.B. Amin, E.P. Sulman, M.P. Lolkema, F.P. Barthel, R.G.W. Verhaak, Radiotheraphy is associated with a deletion mutation that contributes to poor outcomes in patients with cancer. Nat Genet 53 (2021) 1088–1096. https://doi.org/10.1038/s41588-021-00874-3.

23. J. Fares, A.U. Ahmed, M.A. Siddiqui, P.N. Gollavilli, V. Ramesh, B. Parma, A. Schwab, M.E. Balyasnikova, J.P. Chandler, J. Portnow, M.C. Tate, P. Kumthekar, R.V. Lukas, C.D. Hebert, T.V. Strong, C. Amidei, V.A. Arrieta, M.S. Lesniak, Neural stem cell delivery of an oncolytic adenovirus in newly diagnosed malignant glioma: a first-in-human, phase 1, dose-escalation trial, Lancet Oncol 22 (2021) 1103–1114. https://doi.org/10.1016/S1470-2045(21)00245-X.

24. L. Dang, S. Jin, S.M. Su, IDH mutations in glioma and acute myeloid leukemia, Trends Mol Med 16 (2010) 387–397. https://doi.org/10.1016/j.trendsmolmed.2010.07.003.

25. V. Staedtke, O. Dzaye, M. Holdhoff, Actionable molecular biomarkers in primary brain tumors, Trends Cancer 2 (2016) 338–349. https://doi.org/10.1016/j.trecan.2016.06.002.

26. C.M. Ewing, A.M. Ray, E.M. Lange, K.A. Zuhlke, C.M. Robbins, W.D. Tembe, M.H. Kulke, D.C. Chung, Hoxc6 is overexpressed in gastrointestinal carcinomas and interacts with JunD to regulate tumor growth, Gastroenterology 135 (2008) 907–916, 916 e901-902. https://doi.org/10.1053/j.gastro.2008.06.034.

27. Khan IN, Ullah N, Hussein D, Saini KS. Current and emerging biomarkers in tumors of the central nervous system: possible diagnostic, prognostic and therapeutic applications. Semin Cancer Biol. 2018;52:85–102. https://doi.org/10.1016/j.semcancer.2017.07.004.

28. C.M. Ewing, A.M. Ray, E.M. Lange, K.A. Uzhlik, C.M. Robbins, W.D. Tembe, K.E. Wile, S.D. Issacs, D. Johling, Y. Wang, C. Bizoń, G. Yan, M. Gielzak, A.W. Partin, V. Shannumagam, T. Izatt, S. Sinner, D.W. Craig, S.L. Zheng, P.C. Walsh, J.E. Montie, J. Xu, J.D. Carpten, W.B. Isaacs, K.A. Cooney, Germline mutations in HOXB13 and prostate-cancer risk, N Engl J Med 366 (2012) 141–149. https://doi.org/10.1056/NEJMoa1110000.

29. Bell EH, Zhang P, Fisher BJ, Macdonald DR, McClay RJ, Lesper G, Flemming J, Chakraborty AR, Liu Z, Becker AP, Fabian D, Aldape KD, Ashley LS, Werner-Wasik M, Walker EM, Bahary JP, Kwok Y, Yu HM, Laack NN, Schultz CJ, Gray HJ, Robins HI, Mehta MP, Chakravarti A. Association of MGMT promoter methylation status with survival outcomes in patients with high-risk glioma treated with radiotherapy and temozolomide: an analysis from the NRG oncology/RTOG 0424 trial. JAMA Oncol. 2018;4:1405–9. https://doi.org/10.1001/jamaoncol.2018.1977.

30. K. Fuji, E.M. Ooi, P. Tran, N. Ung, K. Thi, A. Trang, B.M. Fong, D.T. Nagasawa, M. Lim, S.K. Maenhout, S. Van Lint, P.U. Emeagi, K. Thieleman, J.L. Aerts, Enhanced suppressive capacity of tumor-infiltrating myeloid-derived suppressor cells compared with their peripheral counterparts, Int J Cancer 134 (2014) 1077–1090. https://doi.org/10.1002/ijc.28449.

31. Sun C, Mezzadra R, Schumacher TN. Regulation and function of the PD-L1 costimulatory checkpoint. Immunity. 2018;48:434–52. https://doi.org/10.1016/j.immuni.2018.03.014.

32. E.K. Nduom, J. Wei, N.K. Yaghil, N. Huang, Y. Kong, K. Gabrusiewicz, X. Liu, S. Zhou, C. Ivan, J.O. Chen, J.K. Burks, G.N. Fuller, G.A. Calin, C.A. Conrad, C. Creasy, K. Rithipichai, L. Radvanyi, A.B. Heimberger, PD-L1 expression and prognostic impact in glioblastoma, Neuro Oncol 18 (2016) 195–205. https://doi.org/10.1093/neuonc/nov172.

33. J. Tel, E.L. Smits, A. Anguille, R.N. Joshi, C.G. Figdor, I.J. de Vries, Human plasmacytoid dendritic cells are equipped with antigen-presenting and tumoricidal capacities, Blood 120 (2012) 3936–3944. https://doi.org/10.1182/blood-2012-06-435941.

34. Y.Q. Yang, W.J. Dong, X.F. Yin, X.S. Xie, J.P. Chen, S.J. Yuan, J.J. Wang, H.X. DeLong, L. Chu, H.N. Xu, X.M. Zhou, R.W. Wang, L. Fang, Y.X. Liu, K.J. Zhang, Interferon-related secretome from direct interaction between immune cells and tumor cells is required for upregulation of PD-L1 in tumor cells, Protein Cell 7 (2016) 536–543. https://doi.org/10.1007/s12298-016-0281-6.

35. Y.C. Ooi, P. Tran, N. Ung, K. Thi, A. Trang, B.M. Fong, D.T. Nagasawa, M. Lim, S.K. Maenhout, S. Van Lint, P.U. Emeagi, K. Thieleman, J.L. Aerts, Enhanced suppressive capacity of tumor-infiltrating myeloid-derived suppressor cells compared with their peripheral counterparts, Int J Cancer 134 (2014) 1077–1090. https://doi.org/10.1002/ijc.28449.

36. K. Maenhout, S. Van Lint, P.U. Emeagi, K. Thieleman, J.L. Aerts, Enhanced suppressive capacity of tumor-infiltrating myeloid-derived suppressor cells compared with their peripheral counterparts, Int J Cancer 134 (2014) 125–132. https://doi.org/10.1002/ijc.28449.

37. K. Ooi, P. Tran, N. Ung, K. Thi, A. Trang, B.M. Fong, D.T. Nagasawa, M. Lim, S.K. Maenhout, S. Van Lint, P.U. Emeagi, K. Thieleman, J.L. Aerts, Enhanced suppressive capacity of tumor-infiltrating myeloid-derived suppressor cells compared with their peripheral counterparts, Int J Cancer 134 (2014) 125–132. https://doi.org/10.1002/ijc.28449.

38. S. Anguille, R.N. Joshi, C.G. Figdor, I.J. de Vries, Human plasmacytoid dendritic cells are equipped with antigen-presenting and tumoricidal capacities, Blood 120 (2012) 3936–3944. https://doi.org/10.1182/blood-2012-06-435941.

39. Y.Q. Yang, W.J. Dong, X.F. Yin, X.S. Xie, J.P. Chen, S.J. Yuan, J.J. Wang, H.X. DeLong, L. Chu, H.N. Xu, X.M. Zhou, R.W. Wang, L. Fang, Y.X. Liu, K.J. Zhang, Interferon-related secretome from direct interaction between immune cells and tumor cells is required for upregulation of PD-L1 in tumor cells, Protein Cell 7 (2016) 536–543. https://doi.org/10.1007/s12298-016-0281-6.

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