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ARTICLE

Prognostic significance of MEOX2 in gliomas

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Abstract

Gliomas are the most common malignant primary tumors in the central nervous system and have variable predictive clinical courses. Glioblastoma, the most aggressive form of glioma, is a complex disease with unsatisfactory therapeutic solutions and a very poor prognosis. Some processes at stake in gliomagenesis have been discovered but little is known about the role of homeobox genes, even though they are highly expressed in gliomas, particularly in glioblastoma. Among them, the transcription factor Mesenchyme Homeobox 2 (MEOX2) had previously been associated with malignant progression and clinical prognosis in lung cancer and hepatocarcinoma but never studied in glioma. The aim of our study was to investigate the clinical significance of MEOX2 in gliomas. We assessed the expression of *MEOX2* according to *IDH1/2* molecular profile and patient survival among three different public datasets: The Cancer Genome Atlas (TCGA), The Chinese Glioma Genome Atlas (CGGA) and the US National Cancer Institute Repository for Molecular Brain Neoplasia Data (Rembrandt). We then evaluated the prognostic significance of MEOX2 protein expression on 112 glioma clinical samples including; 56 *IDH1* wildtype glioblastomas, 7 *IDH1* wild-type lower grade gliomas, 49 *IDH1* mutated lower grade gliomas. Survival rates were estimated by the Kaplan-Meier method followed by uni/multivariate analyses. We demonstrated that MEOX2 was one of the transcription factors most closely associated with overall survival in glioma. Moreover, *MEOX2* expression was associated with *IDH1/2* wildtype molecular subtype and was significantly correlated with overall survival of all gliomas and, more interestingly, in lower grade glioma. To conclude, our results may be the first to provide insight into the clinical significance of MEOX2 in gliomas, which is a factor closely related to patient outcome. MEOX2 could constitute an interesting prognostic biomarker, especially for lower grade glioma.

Introduction

The prediction of clinical behavior, response to therapy, and outcome of glioma is challenging. Despite the past 25 years of research into glioma biology, leading to the discovery of several molecular alterations in lower grade and high grade gliomas, therapy development for adult diffuse glioma remains uneven. Glioblastoma remains the most common and aggressive primary brain tumor with a very poor prognosis with 5-year overall survival rate below 5% [1]. Current treatment involves surgery followed by radiation and temozolomide chemotherapy but tumor recurrence appears inevitable [2]. Classification of gliomas had traditionally been based on histologic features and degrees of malignancy after hematoxylin and eosin staining criteria according to the World Health Organization (WHO) 2007 classification [3]. But this classification has presented

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several shortcomings, particularly major interobserver variability in histological interpretation [4, 5]. More accurate and reproducible criteria were urgently needed to reliably discriminate glioma subtypes and better predict patient outcome. To serve this purpose the revised 2016 WHO classification of tumors of the central nervous system combines histological and molecular features and places molecular biomarkers, such as the isocitrate dehydrogenase 1 and 2 (*IDH1/2*) mutations and the co-deletion of chromosome arms 1p and 19q, in stages [6]. Despite an extended time of molecular investigation of glioma profile, some interesting molecular biomarkers may have been missed, including MEOX2, to the discovery of which the current study is largely devoted.

Mesenchyme Homeobox 2 (MEOX2, also called *GAX*) belongs to the homeobox gene family and has been established as a growth arrest specific homeobox by cycline dependant kinase inhibitor p21 and p16 activation [7]. It is expressed in vascular smooth muscle and vascular endothelial cells [8, 9]. According to a recently published study, *MEOX2* overexpression suppresses proliferation and migration of vascular smooth muscle cells [10]. In addition, *MEOX2* expression could inhibit endothelial cell proliferation and angiogenesis by *NF-kB* down-regulation [11]. In a tumoral context, the dual role of MEOX2 has been reported. In hepatocellular carcinoma and in larynx carcinoma, reduced *MEOX2* expression has been significantly correlated with short overall survival (OS), whereas in lung cancer, *MEOX2* overexpression has been correlated with chemoresistance [12–14]. It has also been described as a potential tumor suppressor gene in Wilms tumor [15]. Another study showed that MEOX2-GLI1 axis was involved in cell viability, cellular proliferation and migration capacity in lung cancer, as well as associated with overall clinical survival and therapy prognosis [16].

MEOX2 gene is located at 7p21.2 locus and gain of chromosome 7 is part of the molecular signature of glioblastoma, especially the classic Verhaak subtype [6, 17]. Another study describes a cohort of 117 mesenchymal glioblastomas and identified a high risk signature of 17 genes comprising *MEOX2* which correlated with overall survival [18]. Another work reported *MEOX2* down-regulation in 15 pools of various cells (glioma stem cell line, astrocytes overexpressing oncogenic and iPSC-inducing factors, conventional glioblastoma cell lines) compared to normal astrocytes [19]. But *MEOX2* expression has not been evaluated in a combined cohort of lower grade/high grade gliomas or according to the WHO 2016 classification. Moreover, its expression in glioma clinical samples and its role in gliomagenesis have never been deciphered.

The aim of our study was to assess MEOX2 expression and its prognostic value in gliomas. We showed that MEOX2 was an interesting prognostic marker in gliomas using a clinical cohort of 112 gliomas and public datasets, namely, The Cancer Genome Atlas (TCGA), The Chinese Glioma Genome Atlas (CGGA) and the US National Cancer Institute Repository for Molecular Brain Neoplasia Data (Rembrandt).

Materials and Methods

Patient cohort, clinical data and molecular traits

A total of 112 patients, operated at the University Hospital of Poitiers with de novo gliomas/glioblastomas diagnosed, were included in this study. The use of human tissue was granted by the secretary of state for education and research, directorate-general for research and innovation, bioethics unit (DC-2008–565), and in accordance with the Declaration of Helsinki. Median age at diagnosis was 51 years and the median survival was 25 months. Patients had received chemotherapy with temozolomide and radiotherapy in 56% of cases (Table 1).

Bioinformatic analyses

Normalized RSEM gene-level RNAseq data, methylation profile (beta value) and Gistic2 thresholded copy number calls of glioblastoma and lower grade glioma cohort from TCGA were downloaded from Broad GDAC Firehose (gdac.broadinstitue.org). Clinical data were obtained from Table S1 of TCGA publication "Molecular Profiling Reveals Biologically Discrete Subsets and Pathways of Progression in Diffuse Glioma" (http://www.sciencedirect.com/science/article/pii/S009286741501692X). Normalized FPKM RNAseq data from the Chinese Glioma Genome Atlas (CGGA) were obtained from http://cgga.org.cn:9091/gliomasdb/download.jsp. The level of *MEOX2* mRNA was classified as low or high according to the mean. Lower grade gliomas *IDH1/2* wildtype subgroup was additionally classified according to quartiles. It has been acknowledged that primary and secondary glioblastomas are distinct tumor entities with distinct molecular features that originate from different precursor cells [20]. Hence, only *IDH1* wild-type glioblastomas were taken into account in this study (for CGGA, TCGA and clinical datasets), as *IDH1* mutated glioblatomas are mostly secondary glioblastomas [21]. Microarray data from the US National Cancer Institute Repository for Molecular Brain Neoplasia Data (REMBRANDT) cohort were acquired from Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc = GSE68848), normalized by RMA and using a custom CDF downloaded from BrainArray (http://brainarray.mbni.med.umich.edu/Brainarray/Database/CustomCDF/

Table 1 Clinical and molecular traits of patients from clinical cohort

		All gliomas	Lower Grade gliomas	Glioblastomas
Number of patients (%)		112 (100)	56 (100)	56 (100)
Gender	Female (%)	44 (39)	24 (43)	20 (36)
	Male (%)	73 (61)	32 (57)	36 (64)
Age	Median (years)	51	40	62
	(Min - Max)	(22–83)	(22–81)	(32–83)
Resection type	Biopsy (%)	22 (20)	9 (16)	13 (23)
	Resection (%)	90 (80)	47 (84)	43 (77)
Treatment ($n = 111$)	Chemo/Radiotherapy (%)	62 (56)	6 (11)	56 (100)
	Chemotherapy (%)	6 (5)	6 (11)	0
	No (%)	43 (39)	43 (78)	0
Survival	Median (Months)	25	44	15
Status	Dead (%)	71 (63)	15 (27)	56 (100)
	Alive (%)	41 (37)	43 (73)	0 (0)
Progression	cases (%)	72 (64)	18 (32)	54 (96)
	Median (Months)	8	26	8
Molecular traits	*IDH1* wildtype (%)	63 (56)	7 (12)	56 (100)
	IDH1 mutated non-codeleted (%)	25 (22)	25 (45)	NA
	IDH1 mutated with 1p/19q codeletion (%)	24 (21)	24 (43)	NA

CDF_download.asp). Z-score normalized expression values of *MEOX2* were taken for the analysis of Ivy Glioblastoma Atlas Project with Allen Institute for Brain Science data sets (http://glioblastoma.alleninstitute.org/static/download.html). RNAseq data of glioblastoma and lower grade glioma cohort from TCGA were analyzed for enrichment of functional gene sets using Gene Set Enrichment Analysis (GSEA, https://software.broadinstitute.org/gsea/index.jsp). C5.bp.v6.1 gene set comprising 4436 GO terms was extracted from the Molecular Signatures Database (MSigDB) in GSEA-compatible GMT format. Only the four most illustrative signatures with Normalized Enrichment Score ≥ 2.74 and a False Discovery Rate < 0.001 were disclosed. *MEOX2* expression network according to TCGA data was performed using RTN and visualized using RedeR and igraph R package installed from Bioconductor (https://www.bioconductor.org/) or CRAN repository (https://cran.r-project.org/web/packages/). Positively regulated genes were illustrated in red, and negatively regulated genes in blue. The length between *MEOX2* and other genes symbolized the strength of the regulation.

Immunohistochemical staining

Tissue micro arrays were established using paraffin-embedded tissue samples (10% neutral buffered) from tumor biopsies or surgical removal of 112 glioma of patients. To overcome tumor heterogeneity, at least 3 biopsy cores of 1 mm diameter and 3 µm thick were included in the recipient block using a tissue microarray workstation (Alphelys, France). For each case, a minimum of 3 cores were transferred from the selected areas to the recipient block, using a tissue microarray workstation (Alphelys, France).

Anti-MEOX2 polyclonal antibody produced in rabbit, from prestige antibodies powered by atlas antibodies, was chosen for immunohistochemistry experiments (Sigma-Aldrich, Missouri, USA). Immunohistochemistry was carried out manually using anti-MEOX2 antibody at 1/500 dilution. Slides were deparaffinized and heated in sodium citrate pH6 solution during 40 min at 96 °C for antigenic retrieval. MEOX2 antibody was incubated overnight at 4 °C and displayed using the streptavidin-biotin-peroxidase method with diaminobenzidine as chromogen (Vectastain® ABC-HRP Kit, Vector laboratories, California, USA). The antibody was validated for immunohistochemistry analysis on placenta sample (39 + 6 months of amenorrhea) and chorionic villi presented the same endothelial cell staining as illustrated in human protein atlas (Figure S1a). Scoring of antibody staining for glioma samples was evaluated by a junior and a senior pathologist independently. Scoring of intensity staining was determined as follows: 0, negative; 1, weak; 2, moderate; and 3, strong (Figure S1b). MEOX2 positive score was settled at score ≥ 1, while negative score at score < 1.

Molecular characterization

Mutations in *IDH1* (R132H, R132C, R132S, R132G) were assessed by Sanger sequencing on a 3500Dx DNA

Sequencer (Applied Biosystems) using the following primers Forward 5-CAGAGAAGCCATTATCTGC-3 and Reverse 5-GGAAATTTCTGGGCCATG-3. The PCR template comprised a denature step lasting 5 mins at 95 °C followed by 35 amplification cycles of 95 °C for 45 s, 56 °C for 45 s, 72 °C for 45 s, and a final extension step at 72 °C for 7 mins. Loss of heterozigosity was evaluated by microsatellite analysis. To analyze the loss of heterozigosity at 1p36 and 19q13 loci, four microsatellite markers on 1p36 (D1S243, D1S199, D1S2734, D1S508) and four microsatellite markers on 19q13 (D19S112, D19S206, D19S412 and D19S596) were used. DNA was amplified and aliquots of the PCR reactions were subjected to electrophoresis and collected data were analyzed with genemapper software (Applied Biosystems). The loss of heterozigosity index was determined with the formula (height (A2/A1) $_{control}$/(A2/A1)$_{tumoral}$). Index < 0.5 and > 1.5 was considered positive.

Statistical analysis

Descriptive statistics of the results were calculated with GraphPad Prism 6 (California, USA). SPSS (IBM, New York) software was used for univariate and multivariates analyses. Statistical significance was evaluated by Kruskal-Wallis, Mann-Whitney, Fisher exact test, Mantel-Cox log rank or Cox regression tests (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). Cox proportional hazard regression model analysis on TCGA dataset was performed using BRB-ArrayTools (NIH). Survival rates were estimated by the Kaplan-Meier method.

Results

MEOX2 is one of the transcription factors most associated with overall survival

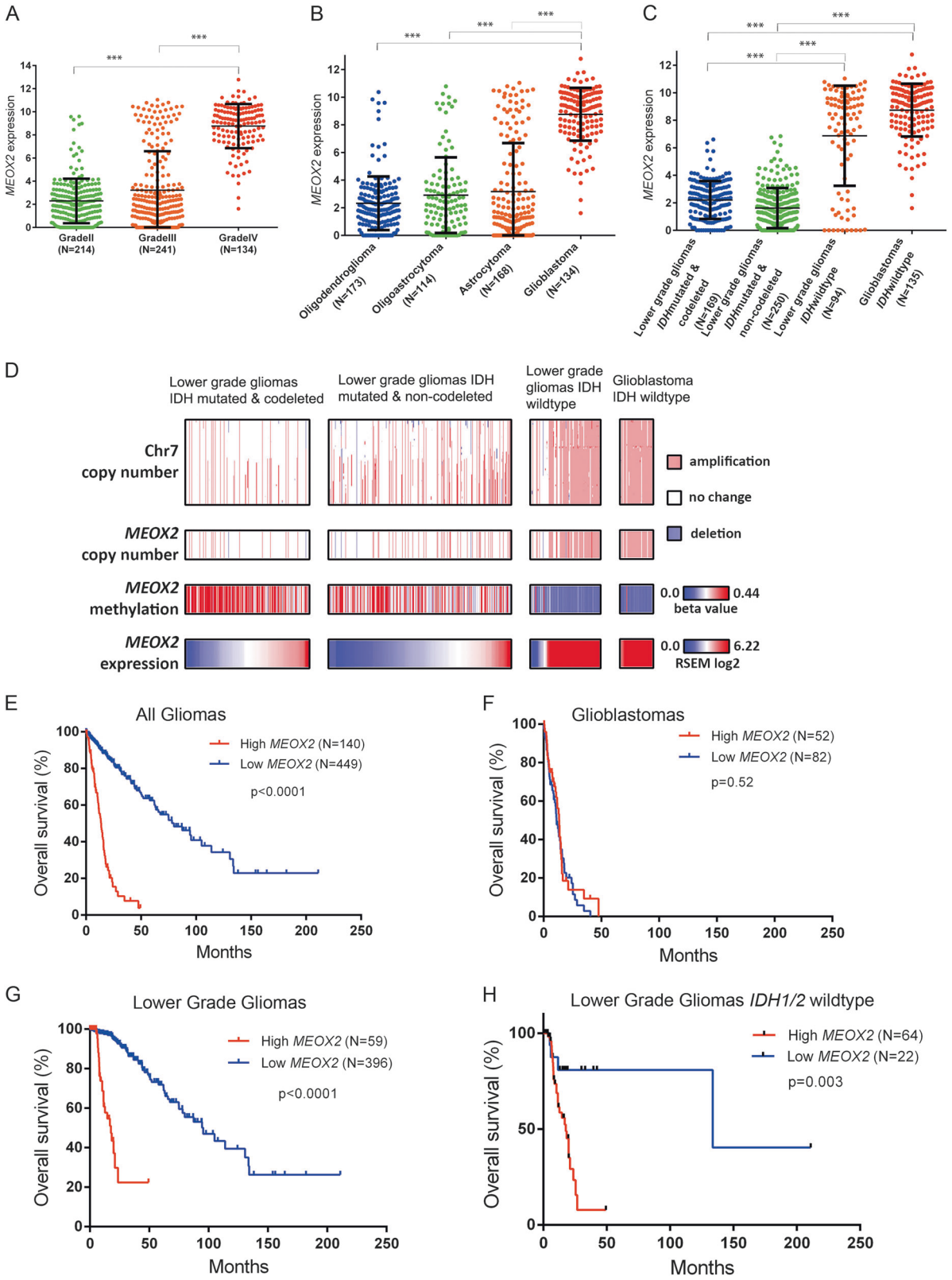
In order to identify new transcription factors associated with the overall survival in gliomas, we performed a Cox proportional hazard analysis on glioblastoma and lower grade glioma RNAseq data from TCGA. Surprisingly, we found that the top ten transcription factor genes encode homeobox proteins (Table 2). We focused our attention on *MEOX2* for which mRNA level was undeniably higher in gliomas than in other human tumors (Figure S2).

MEOX2 is associated with glioma aggressiveness according to TCGA database

The glioblastoma and lower grade glioma cohort comprised 134 glioblastomas (Grade IV) and 455 lower grade gliomas (214 Grade II and 241 Grade III). We showed that *MEOX2* mRNA was more abundant in WHO Grade IV than in Grades II and III ($p < 0.0001$, Fig. 1a). Moreover, *MEOX2* mRNA was enriched in glioblastoma in comparison with other pathological subsets, namely oligodendroglioma, oligoastrocytoma and astrocytoma ($p < 0.0001$, Fig. 1b). More interestingly, we compared the *MEOX2* mRNA level between molecular profiles and found that *IDH1/2* wildtype gliomas (lower grade glioma and glioblastoma) highly expressed *MEOX2* in comparison with *IDH1/2* mutant gliomas ($n = 648$, $p < 0.0001$, Fig. 1c), independently of 1p19q codeletion status. To gain better insight on how this higher *MEOX2* mRNA abundance in *IDH1/2* wildtype tumors could be explained, we analyzed copy number alteration of *MEOX2* and chr7 as well as methylation status of *MEOX2*. As shown in Fig. 1d, amplification of *MEOX2* is strongly associated with chr7 gain. Moreover, a positive correlation was established between *MEOX2* amplification and mRNA level ($r = 0.3287$, $p < 0.001$) (Fig. 1d and S3A). On the contrary, a negative correlation was found between *MEOX2* mRNA abundance and methylation profile ($r = -0.5339$, $p < 0.001$) (Fig. 1d and S3B). Analysis of Kaplan-Meier survival curves of the all glioma cohort showed that a high level of *MEOX2* mRNA was associated with significantly shorter overall survival (14 months versus 80 months, $p < 0.0001$) (Fig. 1e). On the other hand, and

Table 2 The 10 most critical transcription factors highly correlated with patient overall survival in TCGA dataset (Cox proportional hazard analysis)

Rank in Trancription Factor list	Rank in gene list	Symbol	p-value	False discovery rate	Hazard Ratio	Standard deviation
1	27	*HOXA5*	< 1e-07	< 1e-07	1.421	2.996
2	33	*HOXD11*	< 1e-07	< 1e-07	1.413	2.872
3	49	*HOXA3*	< 1e-07	< 1e-07	1.407	3.051
4	59	*HOXA1*	< 1e-07	< 1e-07	1.636	2.244
5	86	*HOXB3*	< 1e-07	< 1e-07	1.348	3.241
6	97	*SHOX2*	< 1e-07	< 1e-07	1.366	3.503
7	111	*HOXD10*	< 1e-07	< 1e-07	1.362	3.007
8	123	*HOXA4*	< 1e-07	< 1e-07	1.357	3.19
9	**132**	***MEOX2***	**< 1e-07**	**< 1e-07**	**1.348**	**3.644**
10	136	*HOXA2*	< 1e-07	< 1e-07	1.402	2.704



contrary to lower grade glioma cohort (Fig. 1g) ($p < 0.0001$), *MEOX2* mRNA was not associated with poor overall survival in glioblastomas (Fig. 1f). Focusing on lower grade gliomas, we performed survival analysis on

◀ **Fig. 1** Expression and clinical relevance of *MEOX2* in TCGA database (**a**, **b**, **c**; Log2). Dot plot representing *MEOX2* expression in TCGA according to histoprognostic grade (**a**), histological type (WHO 2007) (**b**), and WHO 2016 molecular classification (**c**). (**d**) Heat map of GISTIC2 thresholded copy number calls in chr7 and *MEOX2*, methylation status (beta-value) and expression (RSEM log2) of *MEOX2* according to glioma molecular subtypes. (**e-h**) Kaplan-Meier curves plotting overall survival in all gliomas (**e**), glioblastomas (**f**), lower grade gliomas (**g**) and in lower grade gliomas *IDH1/2* wildtype (**h**). Kruskal-Wallis test and Mantel-Cox log rank test were performed to determine the p-value indicated on the panels

each molecular subtype according to the *MEOX2* mRNA mean level and found no significant results (Figure S4). However, we noticed a subgroup in *IDH1/2* wildtype tumors corresponding to the first quartile with a very low *MEOX2* mRNA level similar to *IDH1/2* mutated samples, whereas the remaining major fraction had *MEOX2* mRNA at the same level as glioblastomas (Fig. 1c). More interestingly, this lower quartile of *IDH1/2* wildtype lower grade gliomas showed better overall survival ($p = 0.003$, Fig. 1h). In this subgroup, univariate and multivariate analysis including clinicopathological and molecular features showed that *MEOX2* was an independent prognosis marker ($p = 0.007$ and 0.017 respectively) (Table 3). Interestingly, *MEOX2* mRNA level predicted overall survival more accurately than Chr7gain/Chr10loss, *TERT* expression, *ATRX* mutational status or *MGMT* methylation.

MEOX2 associations confirmed in other public sets CGGA and REMBRANDT

To further confirm our disclosures, we analyzed the results of RNAseq data of 273 tumors from the CGGA dataset and microarray data of 311 gliomas from the REMBRANDT cohort. In both cohorts, we found a positive correlation between *MEOX2* mRNA abundance and tumor grade ($p < 0.0001$, Figure S5A, Figure S6A). As in TCGA cohort, *IDH1* wildtype gliomas highly expressed *MEOX2* in comparison with *IDH1* mutated gliomas ($p < 0.0001$, Figure S5B) and its mRNA expression negatively correlated with overall survival in both CGGA and Rembrandt cohorts ($p < 0.0001$ Figure S5C and Figures S6B). Finally, *MEOX2* mRNA level negatively correlated with survival in lower grade gliomas ($p < 0.0001$), barely or not in glioblastomas, thereby corroborating the previous results showing *MEOX2* as an important marker of progression in lower grade gliomas (Figure S5D, E and S6C, D). Unlike TCGA, the prognostic impact of *MEOX2* in *IDH1* wildtype lower grade gliomas cohort was not significant, probably due to the small number of samples ($n = 49$) (Figure S5F).

Immunohistochemistry investigation confirmed MEOX2 and *IDH1* wildtype glioma association

To further explore the previous findings, clinical and biological data and tumor samples from 112 patients with gliomas were collected. MEOX2 immunohistochemistry analysis on tumor samples transferred into tissue microarrays was carried out. Immunohistochemistry analysis showed the nuclear localization of MEOX2 protein, which was in coherence with its transcription factor function (Fig. 2a). Scoring analysis showed that the highest expression of MEOX2 protein was observed in *IDH1* wildtype gliomas (88% glioblastomas, 11% lower grade gliomas) whereas *IDH1* mutated (100% lower grade gliomas) remained poorly or negatively stained ($p = 0.0152$, Fig. 2b). Combined lower grade gliomas and glioblastomas were consequently separated into two groups according to *IDH1* mutational status. We analyzed the distribution of negative and positive MEOX2 samples according to *IDH1* mutational profile and found that *IDH1* wildtype gliomas comprised 80% of samples with positive MEOX2 staining (Fig. 2c). Among *IDH1* wildtype, 100% of the lower grade gliomas and 77% of glioblastomas were positive for MEOX2 staining. To confirm the prominent distribution of MEOX2 into the most aggressive molecular tumor subtypes, the *IDH1* wildtype tumors, we looked back into the independent cohort dataset. The association between *MEOX2* mRNA expression and aggressive glioma subtypes was even clearer in TCGA dataset. Indeed, only *IDH1/2* wildtype gliomas, comprising 59% glioblastomas and 41% lower grade gliomas, highly expressed *MEOX2* mRNA (Fig. 2d) whereas no *IDH1/2* mutated gliomas did so.

MEOX2 protein expression revealed a prognostic factor in lower grade gliomas

Kaplan-Meier survival analysis of the clinical cohort revealed that positive MEOX2 staining was associated with poor overall survival ($p = 0.009$) and progression-free survival ($p = 0.0078$) (Fig. 3a, b). The same association was observed while analyzing only the lower grade glioma cohort with $p = 0.027$ and $p = 0.078$ for overall survival and progression-free survival respectively, suggesting that MEOX2 could be an interesting prognostic factor (Fig. 3c, d). However, no association was found in glioblastoma for overall survival and progression-free survival (Fig. 3e, f).

Finally, we performed univariate and multivariate analysis including clinicopathological and molecular features. Univariate Cox regression analysis showed an association between MEOX2 protein expression and overall survival ($p = 0.012$) and progression-free survival ($p = 0.009$) in all gliomas (Table 4). In lower grade gliomas, MEOX2 tended

Table 3 Univariate and multivariate analysis of lower grade *IDH1/2* wildtype gliomas of the TCGA dataset (NA: not analyzed, CI: confidence interval)

Lower grade *IDH1/2* wildtype gliomas (n = 86) Variables	Data description		Overall survival			
			Univariate analysis Hazard ratio (CI 95%)	p-value	Multivariate analysis Hazard ratio (CI 95%)	p-value
Age	Mean	51.6	1.056 (1.025–1.088)	< 0.0001	1.054 (1.021–1.087)	.001
Gender	Female	39	0.749 (0.366–1.533)	0.428		
	Male	47				
MGMT promoter status	Methylated	31	0.666 (0.322–1.377)	0.272		
	Unmethylated	55				
Chr 7 gain/Chr 10 loss	Yes	47	0.644 (0.308–1.346)	0.242		
	No	38				
	NA	1				
Chr 19/20 co-gain	Yes	75	0.795 (0.278–2.276)	0.669		
	No	10				
	NA	1				
TERT expression status (log(2) > *2)*	Yes	52	0.462 (0.21–1.016)	0.055		
	No	33				
	NA	1				
ATRX status	Mutant	7	1.055 (0.359–3.1)	0.923		
	Wildtype	79				
MEOX2 expression (*lower quartile*)	High	64	0.187 (0.055–0.634)	0.007	0.222 (0.065–0.761)	0.017
	Low	22				

to be associated with overall survival ($p = 0.058$) and progression-free survival ($p = 0.069$), a finding corroborating survival analysis outcomes (Table 5). Taken together, these results suggested MEOX2 as an interesting prognostic marker in gliomas.

MEOX2 involved in several major carcinogenesis pathways

As *MEOX2* was mainly up-regulated in glioblastoma, we explored its expression according to Verhaak's classification. In TCGA dataset, a significant increase of *MEOX2* mRNA was observed in the classical subtype in comparison with proneural ($p < 0.0001$), mesenchymal ($p < 0.001$) and neural subtypes ($p < 0.01$) (Fig. 4a). *MEOX2* mRNA enrichment in the classic subtype was even clearer in the CGGA dataset (Fig. 4b). Additionally, we explored *MEOX2* mRNA expression over distinct regions of the tumor from the Ivy Glioblastoma Atlas dataset [22]. *MEOX2* mRNA level was significantly higher in tumor cells and infiltrating tumors than in other components of the tumor such as the perinecrotic zone, proliferating vessels or leading edge (Fig. 4c).

We then analyzed the positively and negatively impacted genes associated with *MEOX2* expression in glioblastoma. As expected, we found that marker genes of classical subtype signaling such as Notch (*JAG1*, *LFNG*) and Sonic Hedgehog (*SMO*, *GAS1* and *GLI2*) were mostly positively regulated in *MEOX2* expression network (Figure S7, Table S1).

Finally, we wished to determine in which biological process *MEOX2* was mainly implicated. By performing GSEA analysis on glioblastoma RNAseq data from TCGA using C5.p.v6.1 gene set from the Molecular Signatures Database (MSigDB), several major Go terms appeared (Fig. 4d), all of them involved in replication, recombination and mitosis. Interestingly, *NOS2*, an induced marker in neurosphere glioma cell lines, was highly enriched in glioblastomas with an elevated *MEOX2* mRNA level (Table S2) [23].

Discussion

Glioma is the most common type of intracranial primary tumor comprising different subtypes of which glioblastoma is the most malignant. Based on the previous histopathological classification system, there was a high rate of intra/interobserver variability leading to divergent diagnoses and inexact prognostic outcomes [4, 5]. Fortunately, in May 2016, the latest version of the 2016 WHO Classification was published, providing more accurate stratification than classification based solely on histopathology [6]. Indeed, it introduced molecular markers, i.e. *IDH1/2* mutational status and 1p/19q codeletion, which are recognized worldwide for their high predictive value [24]. Despite the extended time of molecular investigation of glioma profile, to our knowledge *MEOX2* has never been reported even though we have demonstrated that it is an interesting prognostic marker.

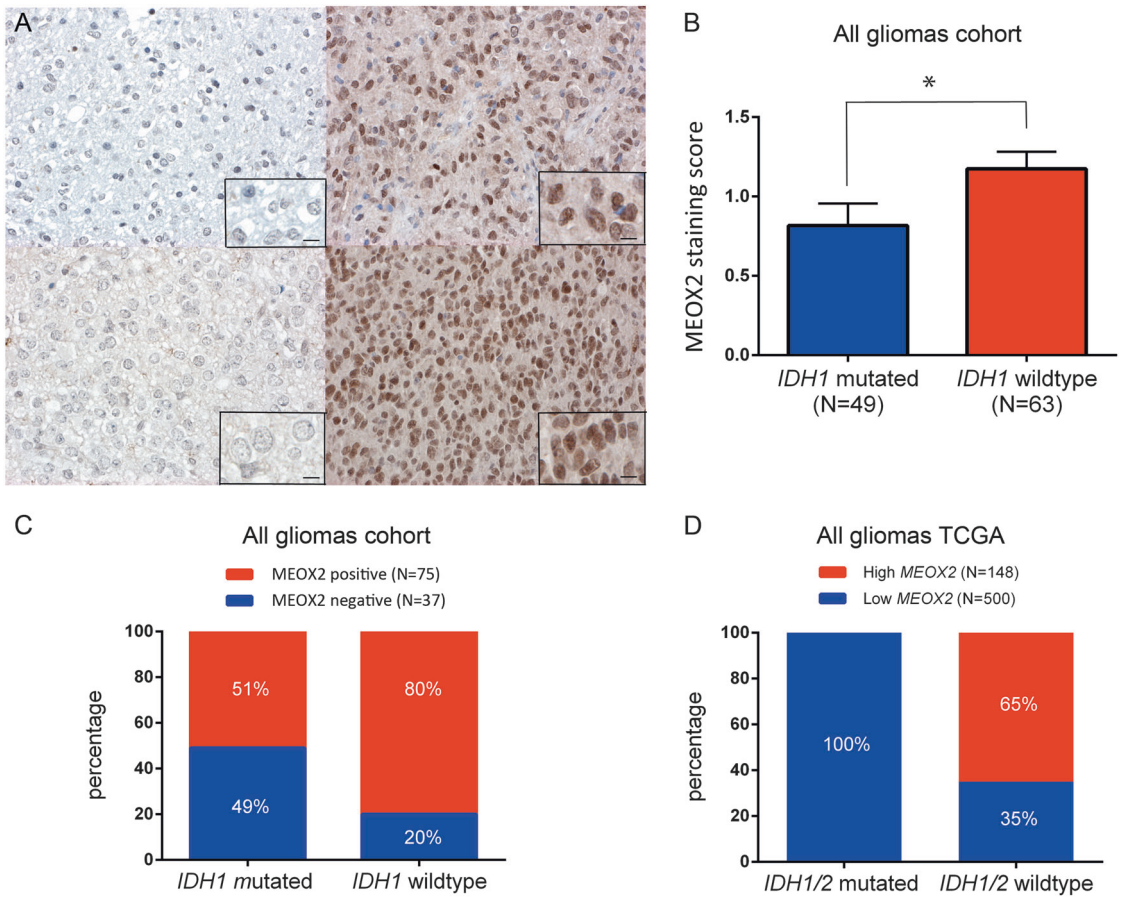


Fig. 2 MEOX2 expression is related to *IDH1* molecular subtypes in TCGA and in clinical data sets. (**a**) Immunohistochemical analysis of MEOX2 protein expression according to *IDH1* mutational status showing the strong nuclear location of MEOX2 in *IDH1* wildtype tumors (right panels (Scale bar: 10 µm). (**b**) Histogram representing the MEOX2 staining scores according to *IDH1* status in the clinical cohort (n = 112, 56 glioblastomas and 56 lower grade gliomas). Histograms represent the mean ± SEM. Mann-Whitney test was performed to determine the statistical significance (p = 0.0152). (**c**) Histogram representing the percentage of negative and positive MEOX2 gliomas according to *IDH1* status. (**d**) In TCGA cohort, histogram representing the percentage of lower and high *MEOX2* gliomas according to *IDH1/2* mutational status

In this study, we identified a new transcription factor of interest in glioma, MEOX2. We showed that MEOX2 is correlated with *IDH1/2* mutational status in public datasets and local clinical datasets. We demonstrated that MEOX2 is a potent prognostic factor of patient outcome in all gliomas and in lower grade gliomas alone. Moreover, it appeared to be a robust prognostic marker of survival in the *IDH1/2* wildtype lower grade glioma subpopulation, independent of the combination of chr7 gain/chr10 loss. Finally, we highlighted replication, recombination and mitosis pathways positively correlated with *MEOX2* up-regulation in glioblastoma.

Several reports have suggested that high expression of homeotic genes, *HOXA9* [25], *HOXD4* [26] and *HOXA13* [27] was an indicator of poor prognosis in glioblastoma patients. We corroborated literature data showing that the ten major transcription factors highly correlated with patient overall survival in gliomas mostly belonged to HOX family. However, the transcription factor MEOX2 which was not a classical HOX family member, has seldom been reported in gliomas, and remains ranked ninth.

MEOX2 is a homeobox protein, a mesodermal transcription factor that plays a key role in somites and limb genesis [28]. In non-tumor tissue, MEOX2 has been described as a cell cycle inhibitor through CDKN1A and CDKN2A activation in endothelial cells [7, 29]. Therefore, it has been described mainly as a negative regulator of angiogenesis and cell proliferation [8]. In tumor tissue, the role of MEOX2 is not clear. *MEOX2* has been reported as a tumor suppressor gene in Wilms syndrome, hepatocellular carcinoma and larynx carcinoma, in which *MEOX2* loss has been correlated with shorter overall survival and poor

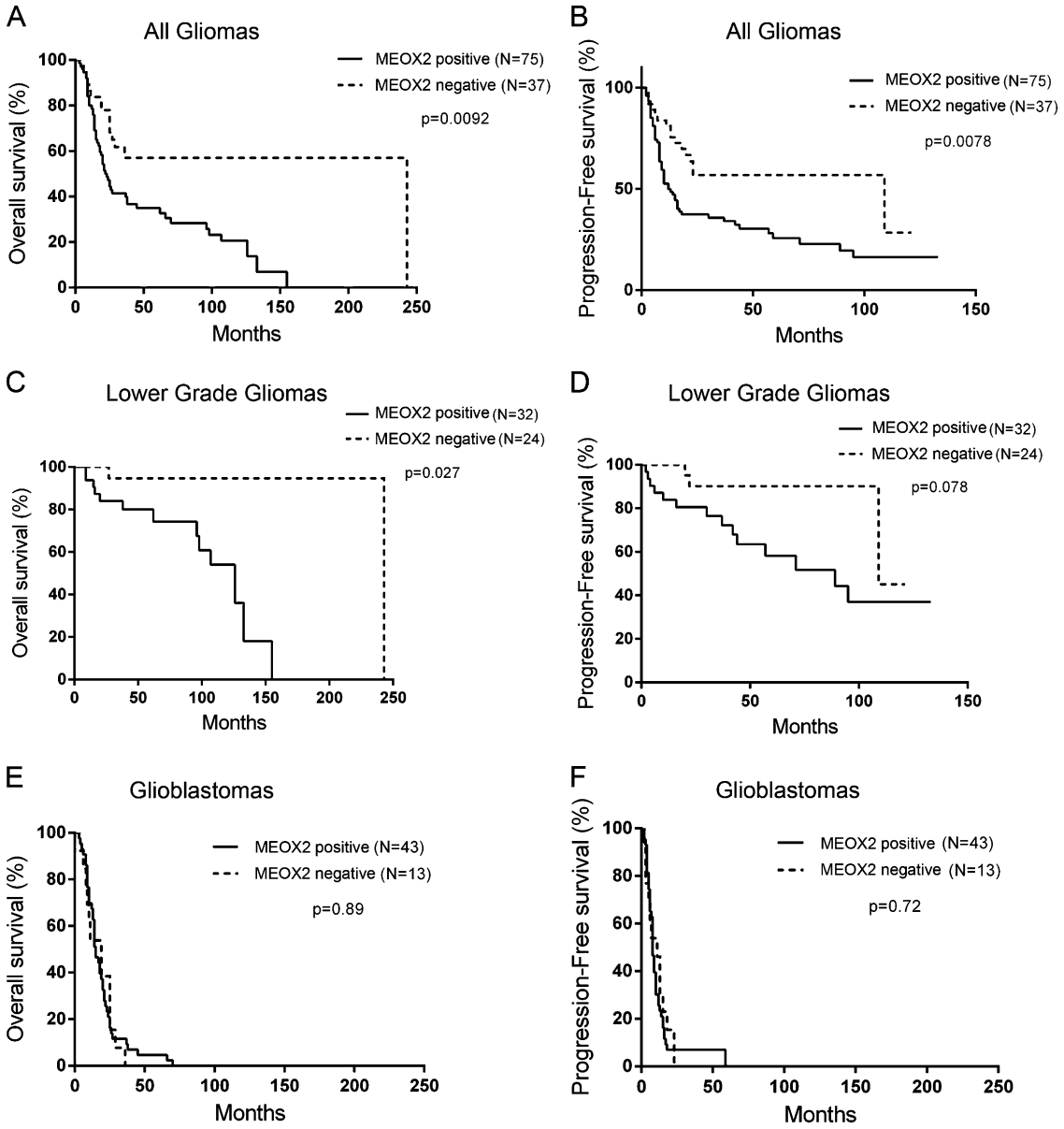


Fig. 3 MEOX2 is associated with poor glioma patient outcome in clinical cohort. (**a-f**) Kaplan-Meier survival curves plotting overall survival or progression-free survival according to MEOX2 staining in all gliomas (**a**, **b**), lower grade gliomas (**c**, **d**) and glioblastomas (**e**, **f**). Mantel-Cox log rank test was performed to determine the p-value indicated on the graphs

disease-free survival [12, 14, 15]. However, *MEOX2* overexpression has been associated with chemoresistance and poor prognosis factor in lung carcinoma [13]. In glioma, conflicting data have also been reported regarding *MEOX2* expression. Bao et al. found a signature of 17 genes including *MEOX2*, which correlated with overall survival in a cohort of mesenchymal glioblastomas [18]. Conversely, Vastrad et al. reported down-regulation of *MEOX2* in various cells compared to normal astrocytes [19]. However, the 15 cell types studied were of diverse origins: glioma stem cell lines, astrocytes overexpressing oncogenic and iPSC-inducing factors and glioblastoma conventional cell lines, and considering them as a group is controversial. None of these studies correlated *MEOX2* expression with glioma molecular profile, either in glioblastomas or in lower grade gliomas. For the first time we showed in several independent cohorts that MEOX2, mRNA and protein, is negatively correlated with progression-free survival and overall survival in gliomas and in lower grade gliomas. Additionally, we demonstrated that *MEOX2* was associated with *IDH1/2*

Table 4 Univariate and multivariate analysis for overall survival and progression-free survival in glioma cohort (CI: confidence interval)

All Gliomas Variables		Overall survival			Progression Free Survival			
		Univariate analysisHazard ratio (CI 95%)	p-value	Multivariate analysisHazard ratio (CI 95%)	p-value	Univariate analysis Hazard ratio (CI 95%)	p-value	Multivariate analysis Hazard ratio (CI 95%)	p-value
Age		1.059 (1.040–1.078)	**< 0.0001**	0.999 (0.971–1.027)	.941	1.046 (1.029–1.063)	**< 0.0001**	1.006 (0.98–1.032)	0.67
Gender	Female	0.697 (0.423–1.148)	0.157			0.784 (0.486–1.265)	0.319		
	Male								
Resection		1.421 (0.799–2528)	0.232			1.663 (0.972–2.845)	0.063		
Treatment		3.194 (2.191–4.655)	**< 0.0001**	1.319 (0.842–2.066)	0.227	2.314 (1.716–3.122)	**< 0.0001**	0.797 (0.540–1.176)	0.253
IDH1 status	Wildtype	38.983 (12.066–125.945)	**< 0.0001**	22.372 (4.905–102.047)	**< 0.0001**	18.183 (8.347–39.608)	**< 0.0001**	13.938 (4.872–39.880)	**< 0.0001**
	Mutant								
Codeletion 1p/19q	Positive	6.14 (2.406–15.669)	**< 0.0001**	2.877 (1.009–8.204)	**0.048**	9.45 (3.32–26.943)	**< 0.0001**	5.4 (1.713–17.025)	**0.004**
	Negative								
MEOX2	Positive	0.47 (0.26–0.847)	**0.012**	1.117 (0.535–2.33)	.768	0.474 (0.271–0.828)	**0.009**	0.989 (0.505–1.937)	0.973
	Negative								

wildtype, a known molecular marker of aggressiveness. Consequently, MEOX2 expression should be considered when assessing the prognosis value of gliomas, particularly lower grade gliomas.

While MEOX2 has been described as an antiangiogenic factor, its function in gliomagenesis is unknown. According to Ivy dataset, *MEOX2* mRNA is confined to tumor cells and tumor infiltrating rather than vascular tissue. Moreover, GSEA analysis supported the idea that *MEOX2* was mainly involved in proliferation, replication and mitosis biological processes. This contrasted with MEOX2 physiological functions in cell cycle arrest [7, 29] and vascular endothelial growth arrest control via NFkB inhibition [11]. The dual function of members of homeobox family has been previously described; they activate growth and migration to promote angiogenesis on the one hand, and to restore or maintain quiescent state on the other hand [30]. Therefore, regarding our results, it seems that the MEOX2 pathways at stake in gliomas are different from the pathways previously described in literature in normal endothelial cells or in hepatocarcinoma tissues.

MEOX2 mRNA was enriched in classical Verhaak subtype and correlated with a chromosome 7 gain and a poor methylation profile. Copy number alteration and decrease of methylation prints at *MEOX2* locus are two mechanisms that could explain, at least to some extent, the elevated level of *MEOX2* mRNA and MEOX2 protein observed in glioblastoma. Interestingly, *CDKN2A*, a known MEOX2 target in endothelial cells, is frequently homologously deleted in the classical subclass, which corroborated the assumption that MEOX2 targeted different genes in glioblastomas than in endothelial cells [31].

One of the upstream effectors that may be responsible for the difference in *MEOX2* mRNA level between the molecular subtypes of glioma could be the IDH1 itself. Indeed, it has been demonstrated that IDH1 R132H mutation induced persistent down-regulation of *MEOX2* in immortalized human astrocytes (IHAs) and patient-derived glioma tumorspheres [32]. Little is known about other possible *MEOX2* regulators. In HUVEC (Human Umbilical Vein Endothelial Cell), microRNA-221 upregulated *MEOX2* through ZEB2 activation, a zinc finger nuclear factor [33]. In human hepatocellular carcinoma and lung adenocarcinoma, down-regulation of microRNA-301 has been shown to be responsible for *MEOX2* activation [34, 35]. To our knowledge, no specific molecule has ever been designed to target MEOX2.

To conclude, our work highlighted a new relevant molecular biomarker in glioma. Further explorations will be needed to define the position of MEOX2 in gliomagenesis and to establish the exact mechanisms responsible for *MEOX2* up-regulation in *IDH1/2* wildtype gliomas.

Table 5 Univariate and multivariate analysis for overall survival and progression-free survival in lower grade glioma and glioblastoma clinical cohorts. (CI: Confidence interval, NA: Not analyzed)

		Lower grade gliomas	Overall survival			Lower grade gliomas	Progression-Free Survival			Glioblastomas	Overall survival	Glioblastomas	Progression-Free Survival
		Univariate analysis	p-value	Multivariate analysis	p-value	Univariate analysis	p-value	Multivariate analysis	p-value	Univariate analysis	p-value	Univariate analysis	p-value
		Hazard ratio (CI 95%)		Hazard ratio (CI 95%)		Hazard ratio (CI 95%)		Hazard ratio (CI 95%)		Hazard ratio (CI 95%)		Hazard ratio (CI 95%)	
Age		1.028 (0.976–1.082)	0.298			1.021 (0.979–1.065)	0.334			1.006 (0.981–1.031)	0.652	0.997 (0.974–1.02)	0.783
Gender	Female	0.422 (0.127–1.404)	0.16			0.966 (0.380–2.458)	0.943			0.925 (0.532–1.607)	0.782	0.649 (0.0.362–1.162)	0.146
	Male												
Resection		0.822 (0.193–3.499)	0.791			2.671 (0.978–7.294)	0.055			1.386 (0.74–2.596)	0.307	0.997 (0.523,1.899)	0.992
Treatment		1.197 (0.645–2.221)	0.568			0.745 (0.378–1.471)	0.397			NA	NA	NA	NA
IDH1 status	Wildtype	17.237 (4.049–73.375)	**<0.0001**	12.835 (2.946–55.926)	**0.001**	16.079 (4.762–54.29)	**<0.0001**	12.472 (3.585–43.383)	**<0.0001**	NA	NA	NA	NA
	Mutant												
Codeletion	Positive	6.8 (1.49–31.164)	**0.013**	4.829 (0.978–23.852)	0.053	21.851 (2.753–173.443)	**0.004**	16.718 (1.995–140.115)	**0.009**	NA	NA	NA	NA
1p/19q	Negative												
MEOX2	Positive	0.138 (0.018–1.070)	0.058			0.313 (0.089–1.092)	0.069			1.043 (0.553–1.966)	0.896	0.897 (0.477–1.688)	0.736
	Negative												

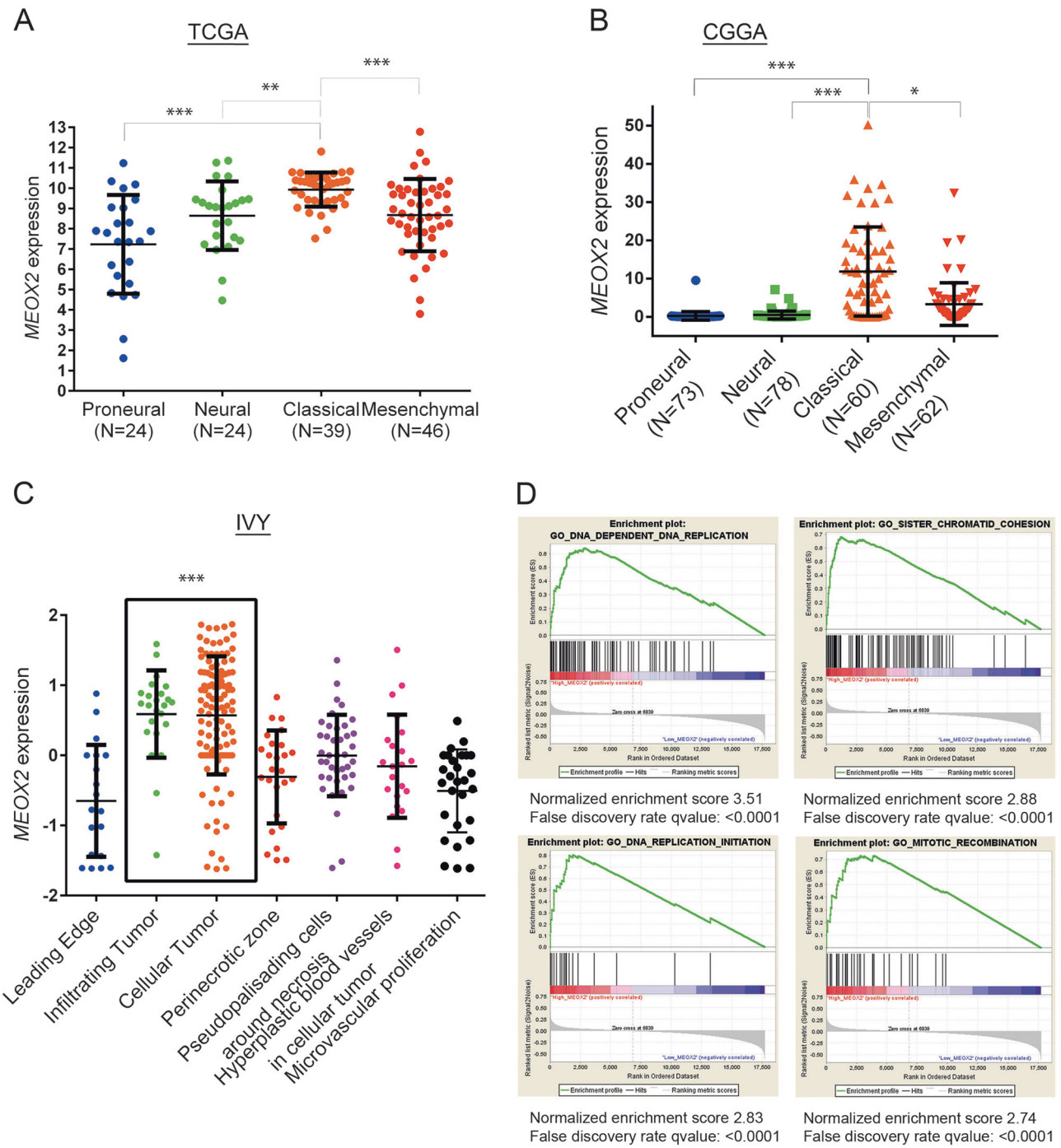


Fig. 4 Major pathways and biological relevance of *MEOX2* in glioblastoma. (**a**, **b**) Dot Plot showing *MEOX2* mRNA expression in the proneural, neural, classical and mesenchymal subtypes of glioblastomas from TCGA (**a**) and CGGA (**b**) datasets. (**c**) Dot Plot showing *MEOX2* mRNA abundance over distinct regions of the tumors from IVY glioblastoma dataset. Kruskal-Wallis test was performed to determine statistical significance. (**d**) The four most illustrative signatures after GSEA analysis in TCGA glioblastoma cohort with Normalized Enrichment Score ≥ 2.74 and False Discovery Rate < 0.0001

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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