mRNA expression levels of hypoxia-induced and stem cell-associated genes in human glioblastoma

MATTHIAS BACHE[1], SWETLANA ROT[1,2], JACQUELINE KESSLER[1], ANTJE GÜTTLER[1], HENRI WICHMANN[1,2], THOMAS GREITHER[3], SVEN WACH[4], HELGE TAUBERT[4], ARIANE SÖLING[5], UDO BILKENROTH[6], MATTHIAS KAPPLER[2] and DIRK VORDERMARK[1]

[1]Department of Radiotherapy, [2]Department of Oral and Maxillofacial Plastic Surgery and [3]Center for Reproductive Medicine and Andrology, Martin Luther University Halle-Wittenberg, Halle; [4]Clinic of Urology, Friedrich Alexander University Erlangen-Nürnberg, Erlangen; [5]Department of Pediatrics, University of Göttingen, Göttingen; [6]Institute of Pathology, Eisleben, Germany

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Abstract. The roles of hypoxia-induced and stem cell-associated genes in the development of malignancy and tumour progression are well known. However, there are a limited number of studies analysing the impact of mRNA expression levels of hypoxia-induced and stem cell-associated genes in the tissues of brain tumours and glioblastoma patients. In this study, tumour tissues from patients with glioblastoma multiforme and tumour adjacent tissues were analysed. We investigated mRNA expression levels of hypoxia-inducible factor-1α (HIF-1α), hypoxia-inducible factor-2α (HIF-2α), carbonic anhydrase 9 (CA9), vascular endothelial growth factor (VEGF), glucose transporter-1 (GLUT-1) and osteopontin (OPN), and stem cell-associated genes survivin, epidermal growth factor receptor (EGFR), human telomerase reverse transcriptase (hTERT), Nanog and octamer binding transcription factor 4 (OCT4) using quantitative real-time polymerase chain reaction (qRT-PCR). Our data revealed higher mRNA expression levels of hypoxia-induced and stem cell-associated genes in tumour tissue than levels in the tumour adjacent tissues in patients with glioblastoma multiforme. A strong positive correlation between the mRNA expression levels of HIF-2α, CA9, VEGF, GLUT-1 and OPN suggests a specific hypoxia-associated profile of mRNA expression in glioblastoma multiforme. Additionally, the results indicate the role of stem-cell-related genes in tumour hypoxia. Kaplan-Maier analysis revealed that high mRNA expression levels of hypoxia-induced markers showed a trend towards shorter overall survival in glioblastoma patients (P=0.061). Our data suggest that mRNA expression levels of hypoxia-induced genes are important tumour markers in patients with glioblastoma multiforme.

Correspondence to: Dr Matthias Bache, Department of Radiotherapy, Faculty of Medicine, Martin-Luther-University Halle-Wittenberg, Ernst-Grube-Str. 40, D-06097 Halle/Saale, Germany
E-mail: matthias.bache@medizin.uni-halle.de

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Introduction

Glioblastoma multiforme is the most common malignant brain tumour and is characterised by a poor prognosis. Glioblastomas often can only be resected partially and a cure is not possible. The treatment for glioblastoma consists mostly of surgery and subsequent radiotherapy with concomitant and sequential chemotherapy. However, despite multimodal treatment, the median overall survival of approximately 10-15 months is still very short (1-3).

Hypoxia is the lack of oxygen, and it is an important factor that reduces the tumour response to radiation and chemotherapy. In addition, tumour hypoxia promotes mutagenesis and leads to genetic instability and clonal expansion of aggressive tumour cell types (4). Numerous studies have shown that hypoxic tumours have a more aggressive phenotype (5). Most high-grade gliomas have hypoxic regions (6). Furthermore, it has been suggested that hypoxia is responsible for malignant progression and the development of 'cancer stem cells' in gliomas (7). *In vitro* analyses revealed that low oxygen levels induced expression of stem cell-associated markers (8-11). It is well known that 'cancer stem cells' in particular are resistant to radiation and chemotherapy.

The prognostic significance of tumour hypoxia markers has been demonstrated in various solid tumours such as head and neck cancer, cervical, breast and prostate cancer, soft tissue sarcoma, melanoma, and glioblastoma (12,13). Furthermore, the contribution of cancer stem cells to the prognosis of glioma patients has been recently discussed (14). There are several therapeutic strategies that affect the expression levels of hypoxia-induced and stem cell-associated genes in glioma (15,16). The impact of mRNA expression levels of hypoxia-induced and stem cell-associated genes in tumour tissue from glioblastoma patients has been insufficiently characterised. However, the most statistically significant gene set identified in a meta-analysis was the hypoxia-inducible

factor (HIF) pathway, which has been repeatedly implicated in glioblastoma (17). In the present study, we determined the impact of mRNA expression levels of different hypoxia-induced [hypoxia-inducible factor-1α (HIF-1α), hypoxia-inducible factor-2α (HIF-2α), carbonic anhydrase 9 (CA9), vascular endothelial growth factor (VEGF), glucose transporter-1 (GLUT-1) and osteopontin (OPN)], and stem cell-associated genes [(survivin, epidermal growth factor receptor (EGFR), human telomerase reverse transcriptase (hTERT), Nanog and octamer-binding transcription factor 4 (OCT4)] that were quantified by quantitative real-time polymerase chain reaction (qRT-PCR) in tumour tissues from patients with glioblastoma. In addition to classical stem cell genes (Nanog and OCT4), mRNA expression levels of inhibitor-of-apoptosis protein survivin, the EGFR, and the catalytic subunit of telomerase hTERT were determined. The association of survivin, EGFR and hTERT in stem cells and/or cancer cells is well discussed. Recently, studies suggest that regulation of expression levels of these stem cell-associated genes is important for the development of glioma stem cells (18-23). In addition, there are first indications, that the analysed stem cell-associated genes are upregulated also by hypoxia.

Materials and methods

Patients and tumour material. Pre-therapeutic tumour samples from 34 patients (treated from 1999 to 2004) with primary glioblastoma and 7 samples of brain tissue adjacent to malignant glioma were analysed in this study. The tumours were classified according to the World Health Organisation guidelines.

Tissue was collected following surgical resection and snap-frozen in liquid nitrogen prior to RNA extraction. The patients in this study have been partially described previously (24). Clinical data, treatment and tumour characteristics were recorded (Table I). The median patient age was 63 years, and the patient ages ranged from 27 to 78 years. There were 16 male and 18 female patients in this study. The median overall survival time was 9.8 months (0.7-47 months). The study was performed in compliance with the Helsinki Declaration and was approved by the Ethics Committee of the Medical Faculty of the University of Halle.

Table I. Clinical characteristics of the patients with glioblastoma.

Characteristics	N	%
Age (years)		
<60	10	29.4
≥60	24	70.6
Gender		
Male	16	47.1
Female	18	52.9
Localization		
Frontal	7	20.6
Parietal	5	14.7
Temporal	13	38.2
Occipital	3	8.8
Other	2	5.9
Not available	4	11.8
Resection		
Total	14	41.2
Partial	16	47.1
Not available	4	11.8
Radiotherapy (median dose: 45 Gy)		
Yes	23	67.6
No	11	32.4
Chemotherapy (BCNU or hydroxyurea)		
Yes	19	55.9
No	15	44.1

Reverse transcription- and quantitative real-time PCR (qRT-PCR). Total RNA from the glioblastoma tissue samples was isolated by the TRIzol method (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. For cDNA synthesis we used 1 μg of RNA in a 'RevertAid H-Minus' First Strand cDNA Synthesis kit (Fermentas, Waltham, MA, USA) as described in the manufacturer's instructions. For qRT-PCR, 1 μl of cDNA was added to the Maxima SYBR Green/ROX qPCR Master Mix (2X), with 20 μM PCR primers (Table II) and distilled water according to the manufacturer's instructions (Fermentas/Thermo Fisher Scientific, Inc.). The qRT-PCR reaction was performed on a Rotor-Gene 6000 (Qiagen, Hilden, Germany) as previously described (25). The PCR cycling included incubation for 15 min at 95˚C, followed by 40 cycles of the following: 30 sec at 95˚C, 30 sec at the specific annealing temperature (Table II) and 30 sec at 72˚C. A melting curve analysis in the temperature range of 65-95˚C (5˚C/sec) was performed. Three genes were used as reference: RNA polymerase II (RPII), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and hypoxanthine-guanine phosphoribosyl transferase (HPRT). Based on the NormFinder software, RPII was the most stably expressed gene in glioblastoma tissue. The expression levels of hypoxia-induced or stem cell-associated genes were quantified as (specific) mRNA copies/RPII mRNA copies.

Statistical methods. All statistical analyses were performed with the SPSS v.19 software package for Windows (SPSS Inc., Chicago, IL, USA). The differences in the numerical data between glioblastoma tissue and tumour adjacent tissue were evaluated using the Mann-Whitney U test. Bivariate correlation analyses were performed by Spearman's rank correlation test. The mRNA expression levels were stratified according to the median into low and high expression groups for each analysed gene. These expression groups were associated with clinicopathological parameters in cross-classified tables (Chi-square test). For survival analyses the overall survival of patients was used as the end point. The survival curves were generated using Kaplan-Meier analysis, and the log-rank test was applied to test for differences. For further univariate analyses, the Cox's proportional-hazards regression model was used to calculate the hazard ratio in the survival analysis. A P-value of <0.05 was considered to indicate a statistically significant result.

Table II. Gene name, primer, annealing temperature, NCBI number and primer localization for qRT-PCR.

Genes	Gene name	Primer	Annealing temperature (˚C)	NCBI number	Primer localization
Reference	HPRT	Sense Antisense	60	NM_000194.2	fw 391-410 rev 652-633
	GAPDH	Sense Antisense	58	NM_002046.5	fw 1055-1074 rev 1164-1145
	RPII	Sense Antisense	60	NM_000937.4	fw 1358-1377 rev 1440-1421
Hypoxia	HIF-1α	Sense Antisense	60	NM_001530.3	fw 1309-1330 rev 1406-1384
	HIF-2α	Sense Antisense	60	NM_001430.4	fw 2521-2540 rev 2737-2718
	VEGF	Sense Antisense	60	NM_001171623.1	fw 1087-1106 rev 1149-1129
	CA9	Sense Antisense	60	NM_001216.2	fw 752-772 rev 880-861
	GLUT-1	Sense Antisense	60	NM_006516.2	fw 1483-1503 rev 1589-1570
	OPN	Sense Antisense	60	NM_001040058.1	fw 610-629 rev 758-739
Stem cell	EGFR	Sense Antisense	60	NM_005228.3	fw 2455-2481 rev 2542-2518
	Survivin	Sense Antisense	60	NM_001168.2	fw 182-203 rev 283-262
	hTERT	Sense Antisense	60	NM_198253.2	fw 1787-1806 rev 1931-1913
	Nanog	Sense Antisense	60	NM_024865.2	fw 343-363 rev 505-483
	OCT4	Sense Antisense	63	NM_002701.5	fw 446-466 rev 629-607

HIF-1α, hypoxia-inducible factor-1α; HIF-2α, hypoxia-inducible factor-2α; CA9, carbonic anhydrase 9; VEGF, vascular endothelial growth factor; GLUT-1, glucose transporter-1; OPN, osteopontin; EGFR, epidermal growth factor receptor; hTERT, human telomerase reverse transcriptase; OCT4, octamer-binding transcription factor 4; RPII, RNA polymerase II; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HPRT, hypoxanthine-guanine phosphoribosyl transferase.

Results

mRNA expression levels of hypoxia-induced and stem cell-associated genes in glioblastoma. The mRNA expression levels of all hypoxia-induced and stem cell-associated genes in tumour tissues from glioblastoma patients (n=34) were higher than in tumour adjacent brain tissues (n=7). A significant difference was detected with respect to hypoxia-induced genes HIF-2α, CA9 and VEGF, and stem cell-associated genes hTERT and OCT4 (Table III). However, the other hypoxia-induced and stem cell-associated genes did not have significant differential expression.

There was a significant association in bivariate analysis (Spearman's rho test) between mRNA expression levels of the hypoxia-induced genes CA9, HIF-2α and GLUT-1 with OPN, a marker of migration and metastasis. Furthermore, the expression of HIF-1α and HIF-2α correlated positively with the stem cell-related gene EGFR. Additionally, the expression of HIF-1α was associated with the expression of the stem cell-associated gene survivin (Table IV). Altogether, our results suggest a distinct hypoxic mRNA profile for glioblastoma. We also found correlations between mRNA levels of stem cell-associated genes. EGFR transcript levels showed a significant positive correlation with survivin, and Nanog mRNA expression was significantly associated with OCT4 expression. However, expression of stem cell-associated and hypoxia-induced genes did not correlate with mRNA levels of hTERT (Table IV).

Association of hypoxia-induced and stem cell-associated gene mRNA levels with clinical characteristics and survival. Based on the median values, we found associations of hypoxia-induced and stem cell-associated gene expression levels with age and gender. Females (n=18) showed higher mRNA expression levels of the hypoxia-induced markers VEGF (P=0.039) and CA9 (P=0.039), and higher expression of the stem cell-associated genes Nanog (P=0.039) and OCT4 (P=0.015) than men (n=16).

Table III. Mean and median mRNA expression levels of hypoxia-induced and stem cell-associated genes in tumour adjacent tissues and glioblastomas.

Gene name	Tissue	Mean mRNA level in copies/copies RPII	Median mRNA level in copies/copies RPII	P-value
OPN	Adjacent	49.96±59.54	16.530	0.061
	Tumour	148.0±147.4	100.700	
HIF-1α	Adjacent	57.51±35.78	35.780	0.083
	Tumour	133.2±129.7	74.800	
HIF-2α	Adjacent	0.832±0.293	0.825	0.035[a]
	Tumour	1.532±0.962	1.393	
CA9	Adjacent	0.001±0.001	0.001	<0.001[a]
	Tumour	0.056±0.082	0.026	
GLUT-1	Adjacent	0.249±0.072	0.249	0.225
	Tumour	0.396±0.288	0.334	
VEGF	Adjacent	0.181±0.224	0.094	<0.001[a]
	Tumour	1.875±1.772	1.349	
Survivin	Adjacent	9.41±10.98	5.600	0.057
	Tumour	23.23±22.25	14.440	
EGFR	Adjacent	32.37±51.82	3.360	0.188
	Tumour	308.9±745.8	12.460	
hTERT	Adjacent	0.001±0.001	0.001	0.049[a]
	Tumour	0.006±0.010	0.002	
Nanog	Adjacent	0.006±0.053	0.003	0.066
	Tumour	0.025±0.043	0.008	
OCT4	Adjacent	0.023±0.026	0.012	0.025[a]
	Tumour	0.088±0.150	0.030	

[a]P-value was considered significant if P<0.05. HIF-1α, hypoxia-inducible factor-1α; HIF-2α, hypoxia-inducible factor-2α; CA9, carbonic anhydrase 9; VEGF, vascular endothelial growth factor; GLUT-1, glucose transporter-1; OPN, osteopontin; EGFR, epidermal growth factor receptor; hTERT, human telomerase reverse transcriptase; OCT4, octamer-binding transcription factor 4.

In addition, higher mRNA expression levels of VEGF (P=0.016) and GLUT-1 (P=0.016) were detected in older patients compared to younger patients. The median age of patients with low mRNA expression levels of VEGF (P=0.016) and GLUT-1 were 9 or 5 years less compared to those patients who had high mRNA transcript levels in their tumours. Based on the median, neither gender nor age showed a significant association with clinical outcome. However, younger patients with an age ≤60 years (n=10) lived longer than patients older than 60 years (P=0.050). In addition, male patients were 9 years younger than female patients (P=0.023).

Kaplan-Meier analyses showed a significant decrease in the overall survival time for glioblastoma patients without radiotherapy and/or chemotherapy (P<0.001). Only 2 of the 9 glioblastoma patients with high mRNA expression levels of hypoxia-associated genes CA9, HIF-2α, GLUT-1 and OPN were treated with radiotherapy and chemotherapy. On the other hand, 16 of the 25 remaining patients (64%) received radiotherapy and chemotherapy (P=0.052). Based on the median levels, glioblastoma patients with high mRNA expression levels of hypoxia-associated genes showed a trend towards shorter survival time. The median survival time of glioblastoma patients with combined high mRNA expression levels of the hypoxia-associated genes CA9, HIF-2α, GLUT-1 and OPN (n=9) was 1.8 months (95% CI, 1.7-1.9 months) vs. 10.1 months (95% CI, 8.1-12.1 months, P=0.061) in the remaining patients (n=25). The risk of an early tumour-related death in patients with high mRNA expression levels of CA9, HIF-2α, GLUT-1 and OPN was 2.13 (P=0.067) (Fig. 1).

Discussion

Hypoxia is a negative prognostic factor in solid tumours including malignant gliomas. A hypoxic microenvironment may promote the self-renewal capability of glioma cells towards a stem cell-like phenotype (8). We analysed mRNA expression levels of different hypoxia-induced and stem cell-associated markers in tumour tissues from 34 patients with glioblastoma. Compared with tumour adjacent brain tissues, the mRNA expression levels of the hypoxia-induced genes HIF-2α, CA9 and VEGF were expressed at a significantly higher level in the tumour tissues (Table III). Our findings are in agreement with the results of our previous study. In our previous study, we examined a limited number of other glioblastomas and

Table IV. Bivariate correlation analysis between mRNA expression levels of hypoxia-induced and stem cell-associated genes in the glioblastomas.

		Hypoxia-induced genes					Stem cell-associated genes				
		HIF-2α	VEGF	CA9	GLUT-1	OPN	EGFR	Survivin	hTERT	OCT4	Nanog
HIF-1α	cor	0.458	-0.015	0.226	0.264	0.130	0.617	0.617	0.057	-0.082	-0.161
	P	0.007[a]	0.931	0.198	0.132	0.465	<0.001[a]	<0.001[a]	0.749	0.643	0.363
HIF-2α	cor		0.407	0.595	0.692	0.518	0.354	0.309	0.048	0.006	-0.109
	P		0.017[a]	<0.001[a]	<0.001[a]	0.002[a]	0.040[a]	0.075	0.786	0.971	0.540
VEGF	cor			0.749	0.696	0.451	-0.063	0.091	0.176	0.281	0.256
	P			<0.001[a]	<0.001[a]	0.007[a]	0.722	0.609	0.320	0.108	0.144
CA9	cor				0.843	0.619	0.165	0.083	0.008	-0.005	-0.110
	P				<0.001[a]	<0.001[a]	0.350	0.641	0.964	0.976	0.536
GLUT-1	cor					0.610	0.275	0.167	0.070	0.003	-0.033
	P					<0.001[a]	0.115	0.344	0.695	0.985	0.855
OPN	cor						-0.014	0.241	-0.115	0.076	0.064
	P						0.938	0.169	0.519	0.671	0.719
EGFR	cor							0.400	-0.001	0.026	0.008
	P							0.019[a]	0.994	0.882	0.966
Survivin	cor								0.098	0.153	0.147
	P								0.582	0.389	0.408
hTERT	cor									0.168	0.217
	P									0.343	0.218
OCT4	cor										0.939
	P										<0.001[a]

[a]P-value was considered significant if P<0.05. cor, correlation coefficient; P, P-value.

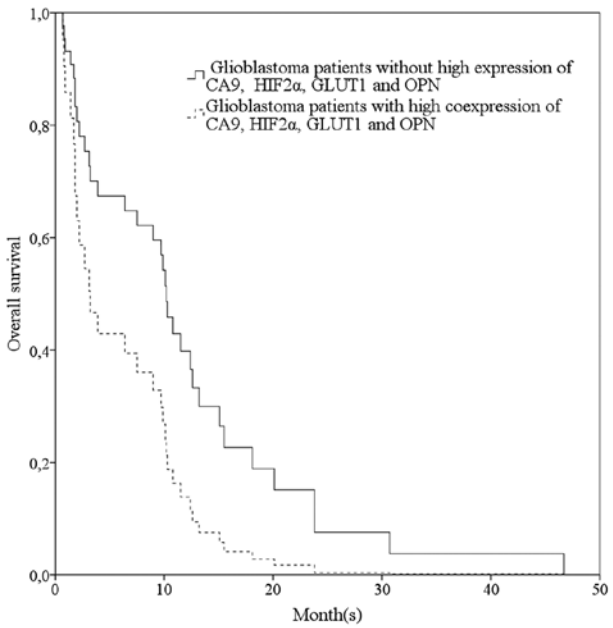

Figure 1. mRNA expression levels of hypoxia-associated genes and overall survival time of glioblastoma patients. Based on the median transcript levels, glioblastoma patients with combined high mRNA expression levels of the hypoxia-associated markers CA9, HIF-2α, GLUT-1 and OPN (dotted line) had, on average, a shorter survival time than the remaining patients (bold line) using a univariate Cox's proportional-hazards regression model. Patients whose tumours weakly expressed hypoxia-associated markers had a 2.13-fold lower risk of an early tumour-related death than patients with high expression of hypoxia-associated markers in their tumours (P=0.067).

detected high mRNA expression levels of HIF-1α, CA9, VEGF and erythropoietin (26). Several other studies have reported higher mRNA expression levels of different hypoxia-induced genes in tissues of glioblastomas compared with low-grade astrocytoma or tumour adjacent tissue (27-30). Other studies detected high mRNA expression levels of stem-cell genes in

malignant glioma compared with surrounding non-tumour tissue (31-35). Our study confirmed these results for the analysed stem cell-associated genes, hTERT and OCT4.

Our analysis showed a strong correlation of expression levels for hypoxia-induced genes with each other. However, the HIF-1α mRNA level was only partially associated with other hypoxia-induced genes. In addition, the transcript levels of hypoxia-induced genes were also significantly associated with high OPN and EGFR mRNA levels in the glioblastoma (Table IV). We previously showed that higher plasma OPN values were detected in glioma patients with larger and more necrotic tumours (36). In addition, a significant association between mRNA expression levels of both hypoxia-induced genes HIF-1α and HIF-2α with the stem cell-related gene EGFR was observed. We also found an association between the expression of HIF-1α and survivin. In patients with glioblastoma, a stem cell-related 'self-renewal' signature was found to be associated with high EGFR expression and therapeutic resistance (37). *In vitro* analyses showed an upregulation of survivin in neuronal and cancer stem cells (21,38). The role of survivin by hypoxia in tumour cells is controversial. However, there is also a link between HIF-1α, survivin and EGFR expression in breast cancer cell lines (39). In addition an association between hypoxia-activated EGFR signaling and the epithelial-to-mesenchymal transition in the epithelial cancer cell line A431 was described (40). Our results suggest that a hypoxia-induced mRNA profile is associated with the expression of stem cell-related genes in glioblastoma tissues. However, the stem cell-associated genes hTERT, OCT4 and Nanog showed no significant association with the expression levels of hypoxia-induced markers. These results indicate that the role of stem cell-related genes in tumour hypoxia should be examined more closely in future studies.

Several studies have demonstrated that higher protein levels of single hypoxia-induced genes correlate positively with malignancy and shorter survival in glioma patients (41-46). However, the prognostic impact is less obvious for hypoxia-induced genes at the mRNA expression level. VEGF but not EGFR, has been shown to be an independent prognostic marker in 86 patients with astrocytic malignant gliomas (30). A study in glioma patients demonstrated that low VEGF mRNA levels were associated with a favourable outcome, but they were not an independent factor in a multivariate analysis (27). However, another study in patients with astrocytoma showed that co-expression of EGFR, IGFBP-2 and HIF-2α were independent prognostic factors (28). This is in agreement with our analysis, which showed that glioblastoma patients with high mRNA expression of CA9, HIF-2α, GLUT-1 and OPN had the shortest median survival time of 1.8 months. Due to the limited patient number in this study, the high co-expression of hypoxia-induced genes showed only a trend of shorter overall survival. Several studies have shown that mRNA expression levels of stem cell markers also predict clinical outcomes and therapy resistance in patients with glioblastoma multiforme (37,47-49). Consistent with these results, the *in vitro* growth potential of patient cancer stem cells correlated with shorter overall and progression-free survival in glioblastoma patients (50). However, in our analysis, the stem cell-associated genes survivin, EGFR, hTERT, Nanog and OCT4 showed no significant association with overall survival. It is possible that protein expression of the analysed stem cell-related genes is more closely associated with survival than mRNA expression.

In summary, in the present study, we detected a link between mRNA levels of hypoxia-induced and stem cell-associated genes in glioblastoma tissues. Both gene groups were expressed at higher levels in glioblastomas than in the tumour adjacent tissues. Notably, elevated mRNA expression levels of hypoxia-induced genes resulted in shorter survival times for glioblastoma patients. Our findings suggest that mRNA expression levels of hypoxia-induced and stem cell-associated genes are important markers in patients with glioblastoma multiforme. However, further prospective studies with a larger number of patients are necessary to support our findings.

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