Pathol. Oncol. Res. (2015) 21:229–240
DOI 10.1007/s12253-014-9813-7

RESEARCH

CXCR7 and *CXCR4* Expressions in Infiltrative Astrocytomas and Their Interactions with *HIF1α* Expression and *IDH1* Mutation

Andre Macedo Bianco · Miyuki Uno · Sueli Mieko Oba-Shinjo · Carlos Afonso Clara · Thais Fernanda de Almeida Galatro · Sergio Rosemberg · Manoel Jacobsen Teixeira · Suely Kazue Nagahashi Marie

Received: 26 August 2013 / Accepted: 4 June 2014 / Published online: 27 June 2014

Abstract The CXCR7, a new receptor for CXCL12 with higher affinity than CXCR4 has raised key issues on glioma cell migration. The aim of this study is to investigate the *CXCR7* mRNA expression in diffuse astrocytomas tissues and to evaluate its interactions with *CXCR4* and *HIF1α* expression and *IDH1* mutation. *CXCR7*, *CXCR4* and *HIF1α* mRNA expression were evaluated in 129 frozen samples of astrocytomas. *IDH1* mutation status was analyzed with gene expressions, matched with clinicopathological parameters and overall survival time. Protein expression was analyzed by immunohistochemistry in different grades of astrocytoma and in glioma cell line (U87MG) by confocal microscopy. There was significant difference in the expression levels of the genes studied between astrocytomas and non-neoplasic (NN) controls ($p<0.001$). AGII showed no significant correlation between *CXCR7/HIF1α* ($p=0.548$); there was significant correlation between *CXCR7/CXCR4* ($p=0.042$) and *CXCR7/IDH1* ($p=0.008$). GBM showed significant correlations between *CXCR7*/*CXCR4* ($p=0.002$), *CXCR7*/*IDH1* ($p<0.001$) and *CXCR7*/*HIF1α* ($p=0.008$). *HIF1α* overexpression was associated with higher expressions of *CXCR7* ($p=0.01$) and *CXCR4* ($p<0.0001$), while *IDH1* mutation was associated with lower *CXCR7* ($p=0.009$) and *CXCR4* ($p=0.0005$) mRNA expressions. Protein expression increased with malignancy and in U87MG cell line was mainly localized in the cellular membrane. *CXCR7* was overexpressed in astrocytoma and correlates with *CXCR4* and *IDH1* in AGII and *CXCR4*, *IDH1* and *HIF1α* in GBM. Overexpression *HIF1α* was related with

A. M. Bianco (✉) · M. Uno · S. M. Oba-Shinjo · T. F. de Almeida Galatro · M. J. Teixeira · S. K. Nagahashi Marie
Department of Neurology, School of Medicine, University of Sao Paulo, Sao Paulo, Brazil Laboratory of Molecular and Cellular Biology, LIM15 Av. Dr. Arnaldo, 455, 4th floor, r.4110, Sao Paulo, SP, Brazil 01246-903
e-mail: abianco@usp.br

A. M. Bianco
e-mail: amb0973@gmail.com

M. Uno
e-mail: unomiyuki99@hotmail.com

S. M. Oba-Shinjo
e-mail: suelimoba@usp.br

T. F. de Almeida Galatro
e-mail: thaisgalatro@hotmail.com

M. J. Teixeira
e-mail: manoelj@netway.com.br

S. K. Nagahashi Marie
e-mail: sknmarie@usp.br

C. A. Clara
Cancer Hospital of Barretos, Pio XII Fundation, State of Sao Paulo, Sao PauloBrazil Rua Antenos Duarte Villela, 1331 BarretosSP, Brazil 14784-400
e-mail: carlosclara@terra.com.br

S. Rosemberg · M. J. Teixeira
Department of Pathology, School of Medicine, University of Sao Paulo, Av. Dr. Arnaldo, 455, Sao Paulo, SP, Brazil 01246-903

S. Rosemberg
e-mail: srosem@uol.com.br

higher expressions of *CXCR7* and *CXCR4*, otherwise *IDH1* mutation related with lower expression of both genes. No association between *CXCR7* and *CXCR4* expression and survival data was related.

Keywords Chemokine · Astrocytoma · CXCR7 · CXCR4 · HIF1α · IDH1

Introduction

Gliomas represent 70 % of primary tumors of the central nervous system (CNS) and 80 % of malignant tumors in young adults (20 to 34 years of age).[1] Diffuse astrocytomas are so designated because these tumors invade the brain tissue by diffuse cellular infiltration and have a significant potential to assume a progressively more malignant biologic behavior over a long period of time. The World Health Organization (WHO) classifies these astrocytomas in three grades based on the histologic features: grade II (diffuse astrocytoma), grade III (anaplastic astrocytoma) and grade IV (glioblastoma).[2] Astrocytomas are the most common and the most aggressive primary brain tumors. The median overall suvival of patients with diffuse astrocytomas is between 3 and 20 years[3–8], whereas the median survival of patients with glioblastoma is approximately 1 year.[9]

Previous reports have shown that chemokines and their receptors play important roles for in tumour growth, metastasis and angiogenesis.[10–12] There is abundant evidence showing that chemokine CXCL12 and its receptor CXCR4, the most frequently overexpressed and best characterized chemokine receptor on tumor cells, are involved in progression of tumors.[13–16] The recent deorphanization of CXCR7 as an additional receptor for CXCL12 with higher affinity than CXCR4 has raised key issues on glioma cell migration.[17, 18] Previous studies showed that the hypoxia-inducible factor 1 (HIF1) transcriptional factor induces CXCL12 and CXCR4 expressions and promoted angiogenesis and cancer cell invasion.[19–23]

The *IDH1* mutation is present in the vast majority of low-grade gliomas and secondary glioblastomas.[24] A recent report showed that mutated *IDH1* does not convert isocitrate into α-ketoglutarate (α-KG) but rather enables IDH1 to convert α-KG into 2-hydroxyglutarate (2HG)[25] that influences a range of cellular programs, affecting the epigenome, the transcriptional programs and the hypoxia-inducible factor.

The link between *IDH1* and *HIF1α* highlights the emerging hypothesis that altered metabolic enzymes contribute to tumor growth by stimulating the HIF1 pathway and tumor angiogenesis.

The expression and prognostic impact of *CXCR7* in gliomas has been investigated in only a few studies, and there are even less data available about the expression of *CXCR7* in astrocytomas.[13, 16, 26] The aim of this study is to investigate the expression of *CXCR7* in a series of diffuse astrocytoma tissues and to evaluate its associated expression with *CXCR4*, *HIF1α* and presence of *IDH1* mutation.

Method

This study was approved by the ethics committee of Department of Neurology, School of Medicine, University of Sao Paulo (0243/09). All patients gave written informed consent for the use of their resected tumor. All data including patient age, sex, date of first symptoms, Karnofsky (KPS) and histological diagnostic according to the WHO classification system were extracted from a combination of clinical and pathological records and outpatient clinic records. All patients were monitored until death or until December 31, 2011.

Tumor samples

Twenty two non-neoplastic tissues from epilepsy surgeries (temporal lobectomy procedures) and 129 frozen samples of diffusely infiltrative astrocytomas (25 AGII, 18 AGIII and 86 GBM) were collected during surgical procedures performed by the Neurosurgery Group of the Department of Neurology, School of Medicine of Sao Paulo University, Brazil. Fresh surgical samples were snap-frozen in liquid nitrogen immediately after surgical removal. A 4 μm thick cryostat section of each sample was obtained for histological assessment under light microscopy after hematoxylin-eosin staining. Necrotic and non-neoplastic areas were removed from the frozen block of tissue by microdissection prior to DNA and RNA extraction.

Total RNA Extraction and cDNA Synthesis

Total RNA was extracted from each sample using the RNeasy Mini Kit (Qiagen Inc, Hilden, Germany). Synthesis of cDNA was performed by conventional reverse transcription, using oligo (dT), random primers (Invitrogen, Carlsbad, CA) for extension, RNase inhibitor and Super Script III according to manufacturer's specifications (Invitrogen). The resulting cDNA was treated with RNase H (GE Healthcare, Piscataway, NJ) and diluted with TE buffer.

Quantitative Real Time PCR (qRT-PCR)

The relative expression of *CXCR7*, *CXCR4*, *IDH1* and *HIF1α* was determined by a quantitative real time PCR (qRT-PCR) using the Syber Green I approach. Primers were designed to

amplify a 93 bp length amplicon with the following set of primers (5′ to 3′): *CXCR7* F: GGTCATTTGATTGCCCGC CT, R: GTTGCATGGCCAGCTGATGT; *CXCR4* F: AATC TTCCTGCCCACCATCTACT, R: GTTGCATGGCCAGC TGATGT; *IDH1* F: GGCTTGTGAGTGGATGGGTAA, R: AGGCCCAGGAACAACAAAATC; and *HIF1α* F: CATC CAAGAAGCCCTAACGTGT, R: CATTTTTCGCTTTCTC TGAGCAT. Standard curves were established to ensure amplification efficiency and analysis of melting curves demonstrated a single peak for the primers. SYBR Green I amplification mixtures (12 μL) contained 3 μL of cDNA, 6 μL of 2X Power SYBR Green I Master Mix (Applied Biosystems) and 3 μL of mixture forward and reverse primers to a final concentration 200 nM. Reactions were run on ABI Prism 7,500 sequence detector (Applied Biosystems). PCR was carried out as follows: 2 min at 50 °C, 10 min period of polymerase activation at 95 °C, and 40 cycles at 95 °C for 15 s, and 60 °C for 1 min. Quantitative data were normalized relative to the internal housekeepings controls: hypoxanthine guanine phosphoribosyltransferase gene (HPRT), beta-glucoronidase gene (GUSβ) and TATA-box binding protein (TBP) according to Valente et al.[27] The geometric mean of the three genes was used for normalized the relative expression analysis. Primer sequences were as follows (5′ to 3′): HPRT F: TGAGGATTTG GAAAGGGTGT, R: GAGCACACAGAGGGCTACAA; GUSβ F: AAAATACGTGGTTGGAGAGCTCATT, R: CCGAGTGAAGATCCCCTTTTTA and TBP F: AGGATA AGAGAGCCACGAACCA, R: CTTGCTGCCAGTCTGG ACTGT. The final primers concentrations were 200 nM for HPRT, TBP and 400 nM for GUSβ. The equation $2^{-\Delta\Delta Ct}$ was applied to calculate the relative expression of genes in tumor samples versus the mean of non-neoplastic tissues where ΔCt = mean Ct gene – geometric mean Ct of housekeeping genes and ΔΔCt = ΔCt tumor – mean ΔCt non-neoplastic tissues.[28] The qRT-PCR runs for each sample were performed in duplicate and repeated whenever the Ct values were not similar. Gene expression was scored according to the astrocytoma grade. For statistical analysis, scores ≥ median values were defined as overexpression.

Previously determined *IDH1* mutational status of 105 samples of astrocytomas, was obtained from the tumor bank maintained by the Department of Neurology, Hospital das Clínicas, School of Medicine of the São Paulo University.[29]

Immunohistochemistry and Immunofluorescence

For immunohistochemical detection of CXCR7, tissue sections were processed and subjected to antigen retrieval. The slides were immersed in 10 mM citrate buffer, pH 6.0, and incubated at 122 °C for 4 min using electric pressure cooker (BioCare Medical, Walnut Greek, USA). The specimes were blocked and then incubated with the primary antibodies at the final dilution of 1:200, at a temperature from 16 to 20 °C for 16 h. The reaction was revealed using a diaminobenzidine commercial kit (Novolink, Novocastra, NewCastle-upon-Tyne, UK) at room temperature, and Harris hematoxylin for nuclear staining. A semi-quantitative score system considering both intensity of staining and percentage of cells was applied as follows: for intensity of staining, 0 = negative, 1 = weak, 2 = mod- erate and 3 = strong; for cell percentage, 0 = no cells stained, 1=10–25 %, 2=26–50 %, 3=51–75 % and 4=76–100 %. Only cases with positive cell staining with scores $2 were considered as positive. Digital photomicrographs of representative fields were captured and processed using PICASA 3 (Google, Mountain View, USA).

The immunofluorescence staining procedure was performed using U87MG cell line fixed with 10 % formalin. After successive washing with 0.05 % Tween (Merck, Darmstadt, Germany) diluted in PBS, cells were incubated in blocking solution containing 5 % goat serum (Sigma), 0.3 % TritonX-100 (Sigma) in PBS and 1 h at ambient temperature over a shaker. The cells were then incubated with primary antibodies diluted in blocking solution at 48 °C for 24 h. After this period, the sections were washed again five times with PBS-Tween and incubated for another 24 h at 48 °C with secondary antibodies diluted in 0.3 % TritonX-100 in PBS by introducing 40.6 - diamidine-2-phenylindole dihydrochloride (Sigma) at 1:10.000 to nuclear staining. Primary antibody used was as follows: CXCR7 (Abcam®, dilution 1:600) and secondary antibodies anti-mouse AlexaFluor 594 (Molecular Probes) used at 1:200. The images were obtained with Leica TCS SP2 confocal system (405 nm diode ARKR 488 nm, 594 nm, Ar, and 633 nm lasers Xn, Munich, Germany) using 20×0.7 or 63×1.2 objectives, with automatic adjustment of the hole to get a airyunit 1 to ensure maximum confocality. Tissue was scanned at a thickness of 2 mm, and images were scanned at 2,048×2,048 pixels.

Statistical Analysis

The Kolmogorov-Smirnov normality test was used to analyse the distribution of the expression data and observational time. Statistical analysis of the distribution of the relative expression by qRT-PCR of the four genes studied in the series was performed by the non-parametric Kruskal-Wallis test followed by Dunn's comparison. Correlation between two gene expression levels was performed using Pearson correlation and Spearman-rho for samples with parametric and non-parametric distribution, respectively. The correlation coefficient $r \geq 0.7$ was

Table 1 Median relative expression in infliltrative astrocytomas

	mRNA				Dunn's test
Gene	NN	AGII	AGIII	GBM	*p*
CXCR7	20,68	70,24	72,89	92,48	$p<0,0001$
CXCR4	19,86	53,48	82,67	95,51	$p<0,0001$
IDH1	16,62	56,32	83,56	95,33	$p<0,0001$
HIF1α	15,00	56,64	102,33	91,72	$p<0,0001$

interpreted as a strong correlation, coefficient $0.3 \leq r < 0.7$ as moderate correlation, $r<0.3$ as weak correlation. R positive values refer to magnitudes proportional, while negative values were interpreted as inversely proportional quantities. Survival curves were made with the Kaplan-Meier method and differences in survival analyzed for significance by the log-rank test. Overall survival (OS) time was calculated as the interval between the surgery and day of death, in months.

All statistical calculations were performed using SPSS 20 software (SPSS, Chicago, IL, USA), with $p<0.05$ being considered statistically significant.

Results

CXCR7, *CXCR4*, *IDH1* and *HIF1α* mRNA Expressions in Surgical Astrocytoma Specimens

The median expression of *CXCR7*, *CXCR4*, *IDH1* and *HIF1α* mRNA were showed in Table 1.

The median values of *CXCR7*, *CXCR4*, *IDH1* and *HIF1α* showed significant difference between astrocytoma (AGII, AGIII) and GBM samples (*** $p<0.0001$). The median *CXCR4* mRNA expression between GBM and AGII was also significantly different (** $p=0.0029$)

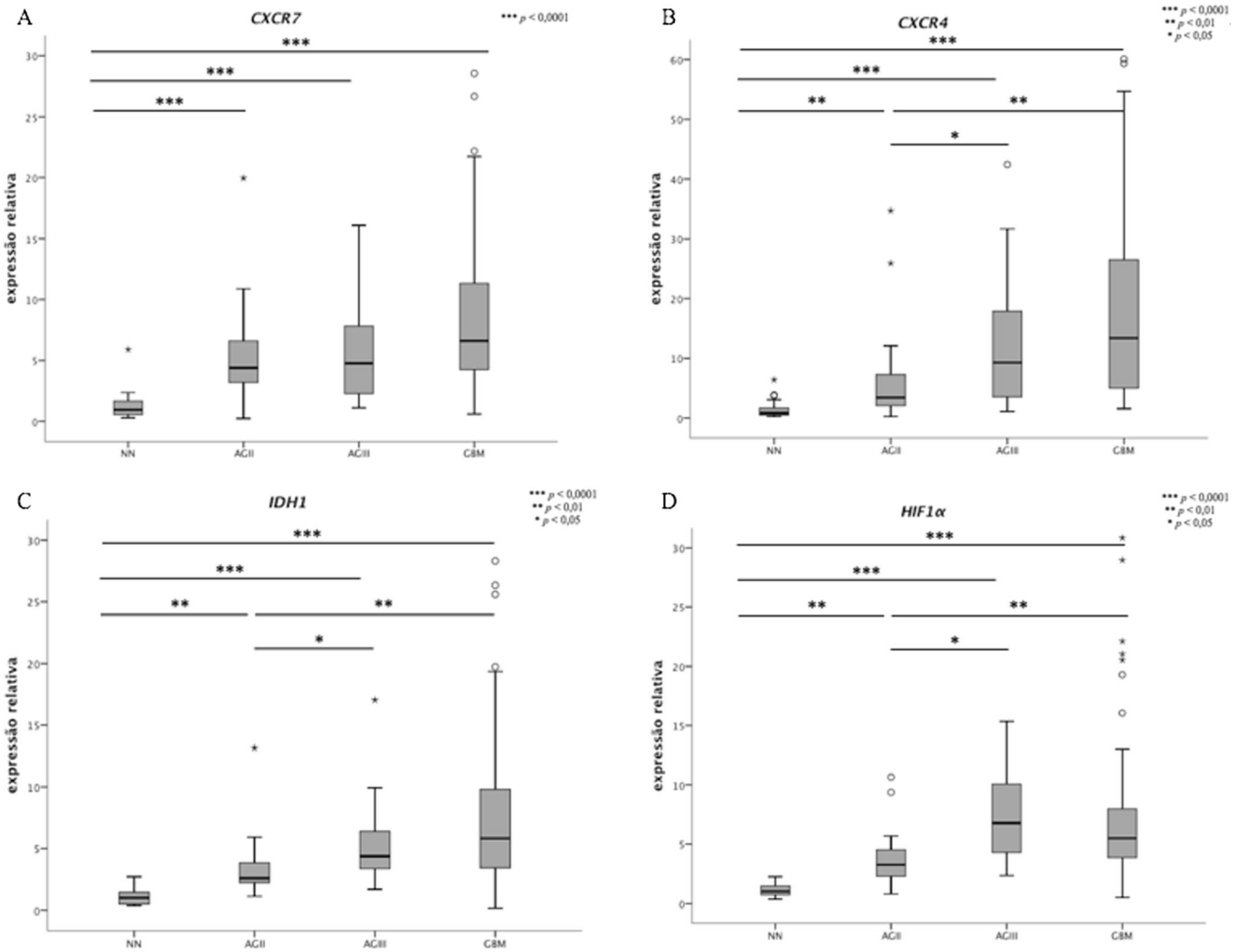

Fig. 1 **a** *CXCR7,* **b** *CXCR4,* **c** *IDH1* and **d** *HIF1α* mRNA expression in 22 non-neoplastic tissues, 25 AGII, 18 AGIII and 86 GBM assessed by qRT-PCR. Higher expression levels were observed in tumor

AGIII and GBM) and NN brain tissue ($p<0.0001$). Higher expression levels were observed in tumor samples. The *CXCR4, IDH1* and *HIF1α* mRNA expression also was significantly different between AGII-AGIII and AGII-GBM Fig. 1.

Associated mRNA Expressions Among *CXCR7, CXCR4, IDH1* and *HIF1α*

In AGII, we observed a significant correlation between *CXCR7 and IDH1* expression ($r=0.52$, $p=0.008$) and

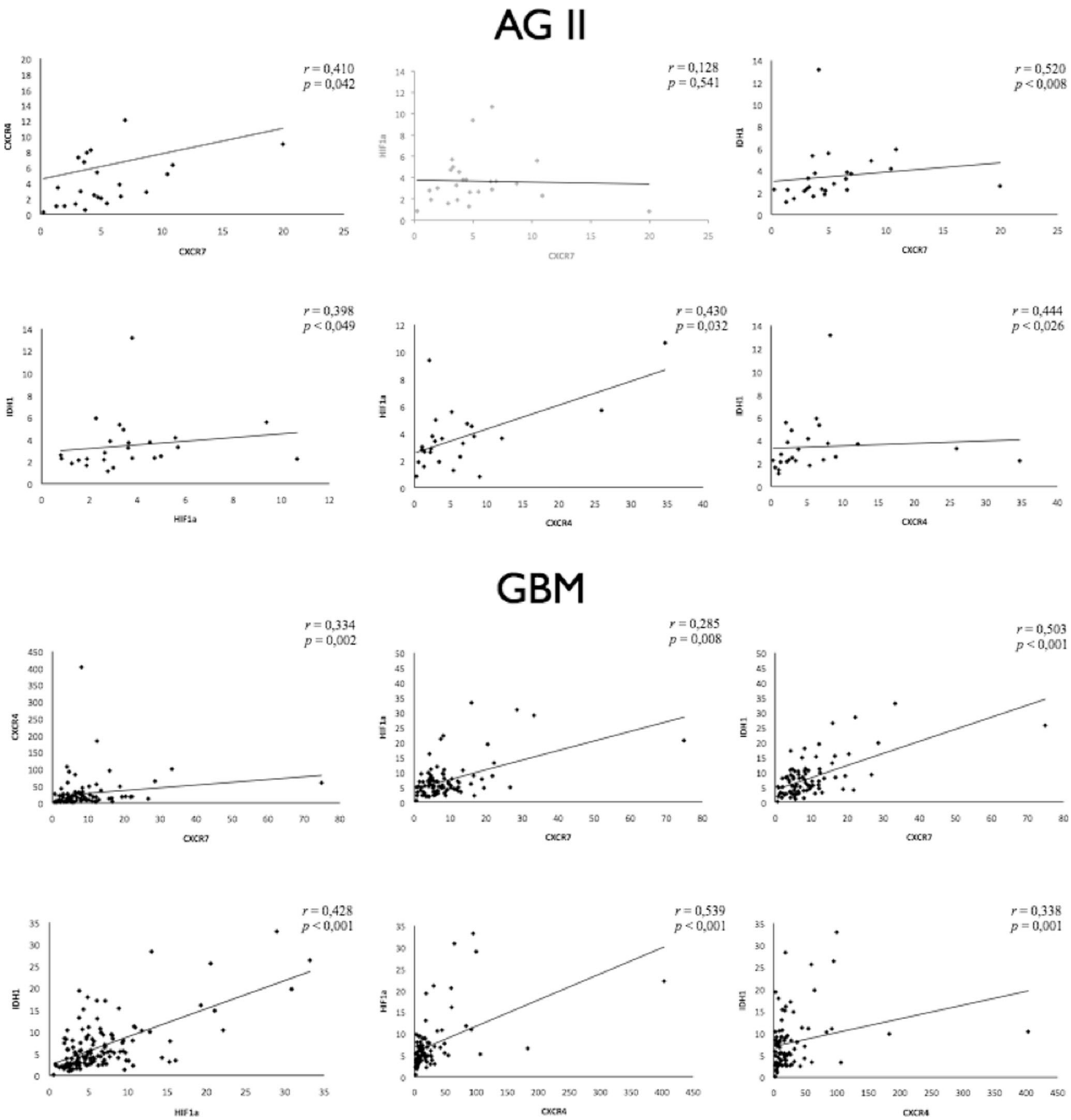

Fig. 2 Correlation between *CXCR7* and *CXCR4*, *HIF1α* and *IDH1* expression levels in diffusely infiltrative astrocytomas. Correlation was assessed in AGII and GBM cases. *CXCR7* expression level was correlated to *CXCR4* and *IDH1* in AGII and with *CXCR4*, *IDH1* and *HIF1α* expression levels in GBM. The significant correlations are shown in black and the non-significant in grey. r correlation coefficient assessed by Spearman-rho test

also between *CXCR7* and *CXCR4* (r=0.41, p=0.042). However, no correlation was identified between the expressions of *CXCR7* and *HIF1α* (r=0.12, p=0.541).

In GBM, on the other hand, a significant correlation between *CXCR7* and *IDH1* (r=0.50, p<0.0001) was observed. Also a correlation between *CXCR7* and *CXCR4* expressions (r=0.33, p=0.002), and a correlation between *CXCR7* and *HIF1α* expressions (r=0.28, p=0.008) were observed. Figure 2.

HIF1α Overexpression Associates with *CXCR7* and *CXCR4* Overexpressions

Patients with *HIF1α* hyperexpression presented higher *CXCR7* (7.94) and *CXCR4* (16.32) mRNA expressions than patients presenting *HIF1α* downregulated, who showed *CXCR7* mRNA median expression of 5.25 (p=0.01) and *CXCR4* of 6.54 (p<0.0001). Fig. 3.

IDH1 Mutation Status and Relative Median Expressions of *CXCR7*, *CXCR4* and *HIF1α*

IDH1 mutation status was analyzed in 105 samples of diffuse infiltrative astrocytomas (19 AGII, 12 AGIII and 74 GBM). The R132H mutation was found in 30 samples distributed as follows: 16 AGII, 6 AGIII and 8 GBM. Patientes with *IDH1wild* and *IDH1mut* presented *CXCR7* expression of 5.91 and 4.26 (p=0.009); *CXCR4* expression of 13.23 and 4.66 (p<0.01); and *HIF1α* expression of 5.66 and 4.61(p=0.346) respectively. Figure 4. The overview of *IDH1* mutation status and relative gene expression for AGII, AGIII and GBM are displayed as heatmap. Figure 5.

Immunohistochemistry and Immunofluorescence

The analysis of CXCR7 protein by immunohistochemistry staining was performed in 6 NN, 6 AGII, 6 AGIII and 7 GBM samples. The protein expression was identified in all selected samples. In AGII, CXCR7 staining was present predominantly in tumor cells and almost absent from the endothelium of the tumor vasculature. In AGIII there was moderate labeling of the tumor cells and more intense labeling of the tumor vascular endothelial cells. While in GBM, there was intense staining of the tumor cells mainly in pseudopalisades nearest to necrotic areas and strong ataining of the tumor endothelium. Figure 6.

Staining performed on the established cell line GBM (U87MG) showed protein expression located mostly in the cytoplasm, with minimal nuclear staining. Analysis of confocal microscopy showed that CXCR7 was primarily associated with the plasma membrane. Figure 7.

Overall Survival (OS)

OS in GBM patients had no significant differences for *CXCR7* (p=0.866), *CXCR4* (p=0.228), *IDH1* (p=0.553) or *HIF1α* (p=0.767) mRNA expression levels comparing those above and under the median expression value for each gene; or when

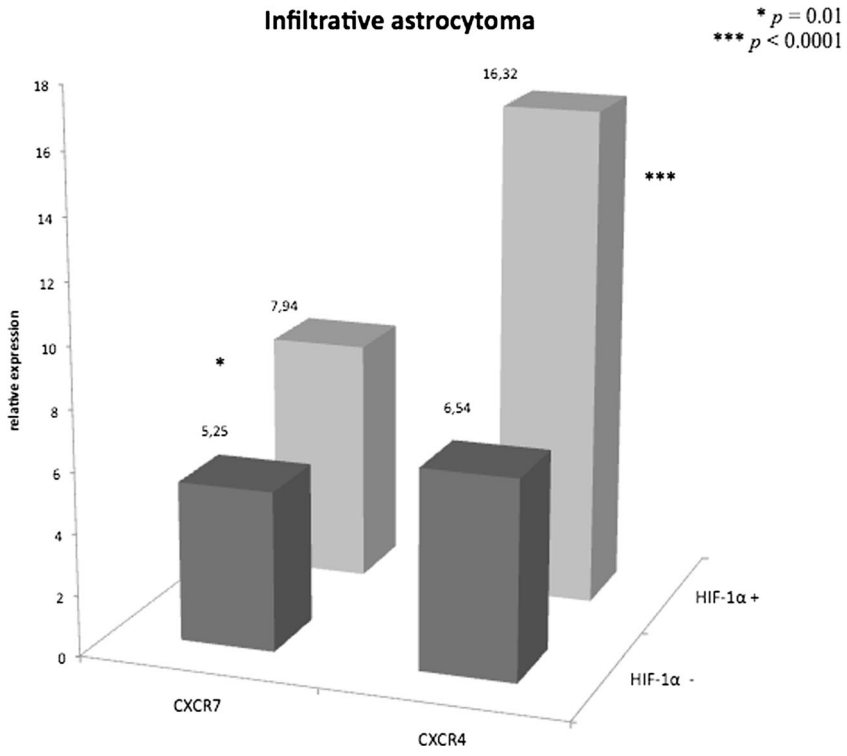

Fig. 3 Impact of *HIF1α* in *CXCR7* and *CXCR4* mRNA expression showing a higher value *CXCR7* and *CXCR4* in patients with overexpression of *HIF1α*

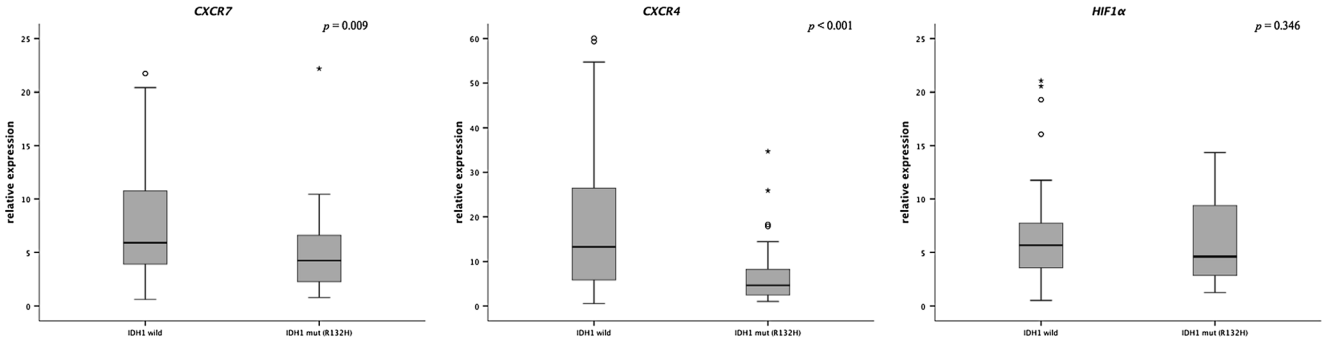

Fig. 4 **a** *CXCR7,* **b** *CXCR4* and **c** *HIF1α* mRNA expression according *IDH1* mutation status. Patients with *IDH1*mut presented lower *CXCR7* and *CXCR4* expressions than *IDH1*wild

assessed in pairs comparing patients presenting hyperexpression of both genes with patients hypoexpressing these genes as: $CXCR7^{+}/CXCR4^{+}$ *vs* $CXCR7^{-}/CXCR4^{-}$ (p=0.395), $CXCR7^{+}/IDH1^{+}$ vs $CXCR7^{-}/IDH1^{-}$ (p=0.052) or $CXCR7^{+}/HIF1\alpha^{+}$ *vs* $CXCR7^{-}/HIF1\alpha^{-}$ (p=0.625). Fig. 8.

Discussion

Tumor microenvironment has proved increasingly relevant in the tumorigenic process and their components have been the focus of study as potential therapeutic targets adjuvants. The results of this study demonstrated that the chemokine receptors, CXCR4 and CXCR7, exhibit differential expression in diffusely infiltrating astrocytomas and their expressions associated with other factors such as *HIF1α* and *IDH1* may favor the process of malignant tumor.

The *CXCR4* and *CXCR7* mRNA expressions were significantly increased in astrocytomas compared to non-neoplastic brain tissue and a increase also was observed in parallel with malignancy. It was observed a significant association with *CXCR7* and *CXCR4* in AGII and in GBM. These findings corroborate previous descriptions in the literature as discussed below.

CXCR4 is the best characterized chemokine receptor in tumor cells and also is the only chemokine receptor identified on cancer stem cells (CSC). $CXCR4^{+}$ CSC have been isolated from GBM and pancreatic carcinoma.[30, 31] CXCR7 is a recently deorphanized receptor for the chemokines CXCL12 (SDF-1) and CXCL11 (I-TAC).[17, 18] The chemokine receptors CXCR7 and CXCR4 contribute to tumorigenesis and play important roles in several common malignancies.[14, 15, 21, 32, 33]

The *CXCR7* is highly expressed in prostate tumors and the amount of expressed protein has been associated with tumor aggressiveness.[34] In breast and lung cancers, the CXCR7 promotes tumor growth.[35] Higher expression of CXCR7 has been associated with early and metastatic recurrence in non-small cell lung cancer.[36] Increased *CXCR7* expression was also found in hepatocellular carcinoma tissues and it was related to invasion, angiogenesis and tumor growth.[37] High levels of *CXCL12* expression were associated with poor overall and disease-free survival in patients with pancreatic ductal adenocarcinoma.[38] Concomitant CXCR4 and CXCR7 expressions have predicted poor prognosis in renal cancer.[14] High levels of CXCR4, CXCR7 and SDF-1 were associated with poor overall survival and recurrence-free survival in renal cell carcinoma patients.[39]

The CXCR7 protein expression showed a range from weakly positive in normal brain tissue, including endothelial cells, to a progressive immunostaining increase both in tumor and endothelial cells in parallel to the increase of malignancy. In GBM more intense labeling occurred primarily in pseudopalisading cells nearest necrosis and highly proliferating tumor endothelium. Similar results were found in the literature from other tumors such as bladder, pancreas, and kidney.[15, 40, 41] But is partly different from those available, witch CXCR7 were found primarily in endothelial cells of

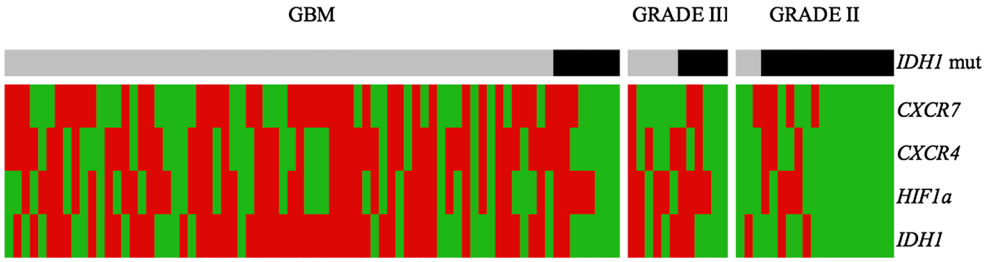

Fig. 5 Heatmap displaying the relative gene expressions in astrocytomas (grade II, grade III and GBM) cases according to *IDH1* mutation status. Samples with an *IDH1* mutation are indicated by black lines and samples with no *IDH1* mutation are indicated by gray lines. Each mark was code as red if overexpression and green if downregulated. The *IDH1*mut cases showed lower expression levels of *CXCR7* and *CXCR4* mRNA

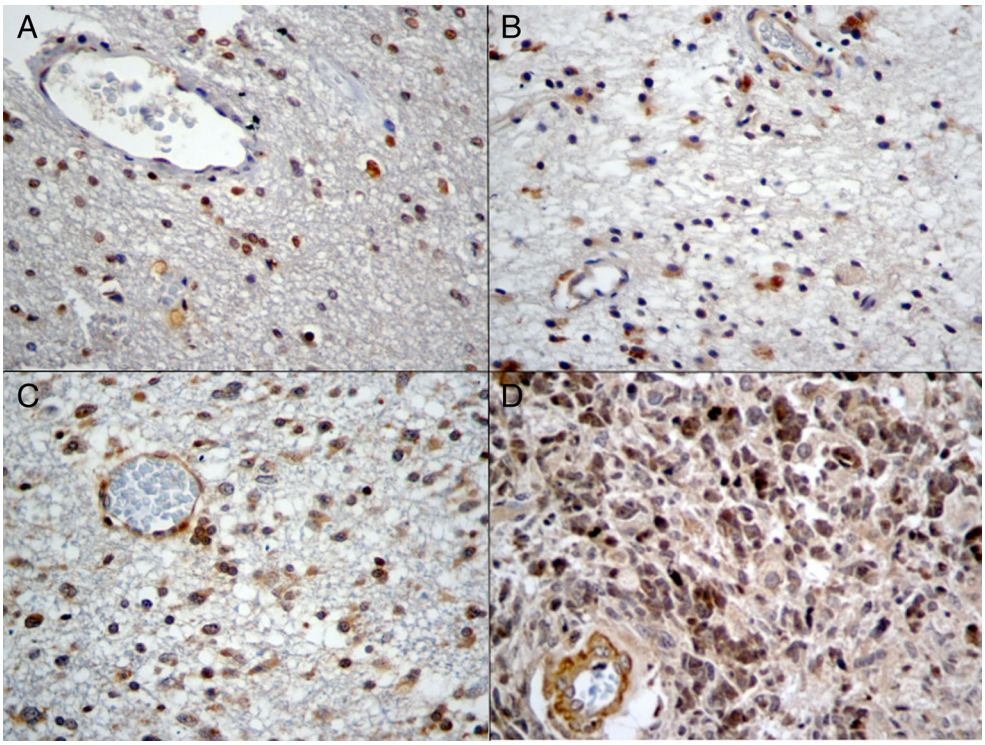

Fig. 6 Immunohistochemistry of CXCR7. Representative examples of NN tissue sample with weak nuclear staining and absence of endothelial marking; AGII - tumors cells presenting predominantly cytoplasmatic staining, and weak labeling of tumor endothelium; AGIII - tumor cells with cytoplasmatic and nuclear staining and moderate labeling of tumor endothelium, and GBM - atypical tumor cells and proliferated endothelium cells presenting strong labeling (IHC 400x)

normal brain and particularly in GBM tumor cells.[16] In other different study CXCR7 was present in tumor cells of GBM and weakly in endothelium, nevertheless no staining was detected in low-grade glioma.[13] The inconsistency data regarding the location of CXCR7 may be justified due to the small number of samples analyzed in these previous studies, unlike observed in a study of cancer of the cervix where CXCR7 was related to tumor size, lymph node involvement

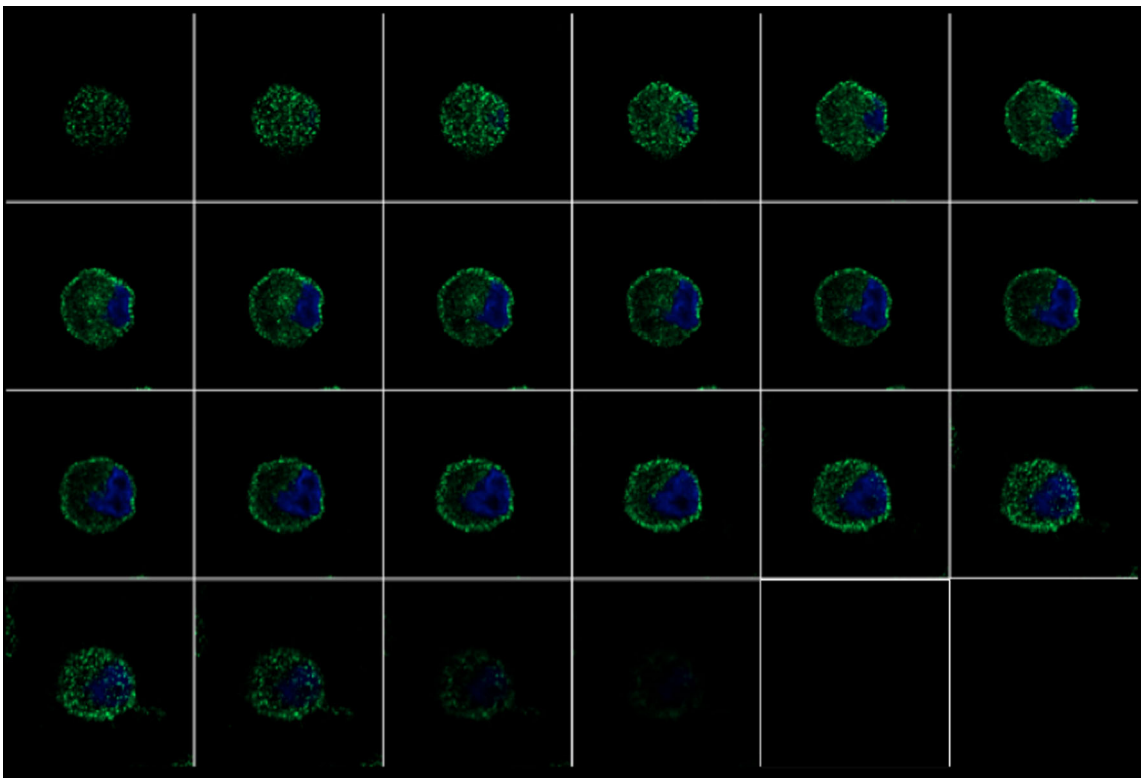

Fig. 7 Confocal microscopy of CXCR7 (*green fluorescence*) in U87MG. DAPI was used for nuclear detection

and disease-free interval.[42] Interestingly GBM endothelial cells overexpressing CXCR7 and CXCL12 but not CXCR4.[43]

The presence of CXCR7 protein on the cell membrane was showed unequivocally in U87MG cell line, as well as in the cytoplasm and also a thin marking at the core of some tumor cells. It's possible to speculate that the cell membrane receptor is internalized and there is protein traffic to the nucleus, corroborated by the observation of nuclear staining in the surgical samples. Description of similar traffic from the cell surface to the nucleus has been described for EGFR[44–46], can be inferred by analogy to similar mechanisms for CXCR7. This observation deserves to be further investigated in future studies.

Hattermann et al.[16] showed that *CXCR7* is highly expressed *in situ* on tumor endothelial, microglial and glioma cells whereas *CXCR4* has a much more restricted localization. These authors also demonstrated that *CXCR7* transcription significantly increased in high-grade astrocytomas as compared with normal brain tissues, and that CSC line preferentially expressed CXCR4, which diminished upon differentiation, whereas CXCR7 increased drastically with differentiation. However, Calatozzolo et al.[13] showed significantly higher *CXCL11* and *CXCR4* mRNA expressions in GBM specimens compared to non-tumor controls or low-grade gliomas, but reported no differencial expression for *CXCL12* and *CXCR7*.

In the present study, we observed a significant increase of *CXCR7* expression in astrocytomas when compared to non-neoplastic brain tissue together with significant increase of *CXCR4*, *IDH1* and *HIF1α* expressions. Of note, a significant differential *CXCR4* expression was detected between AGII and GBM that was two fold higher in GBM. In AGII we showed a significante associated expression of *CXCR7/CXCR4* and *CXCR7/IDH1,* while no association between

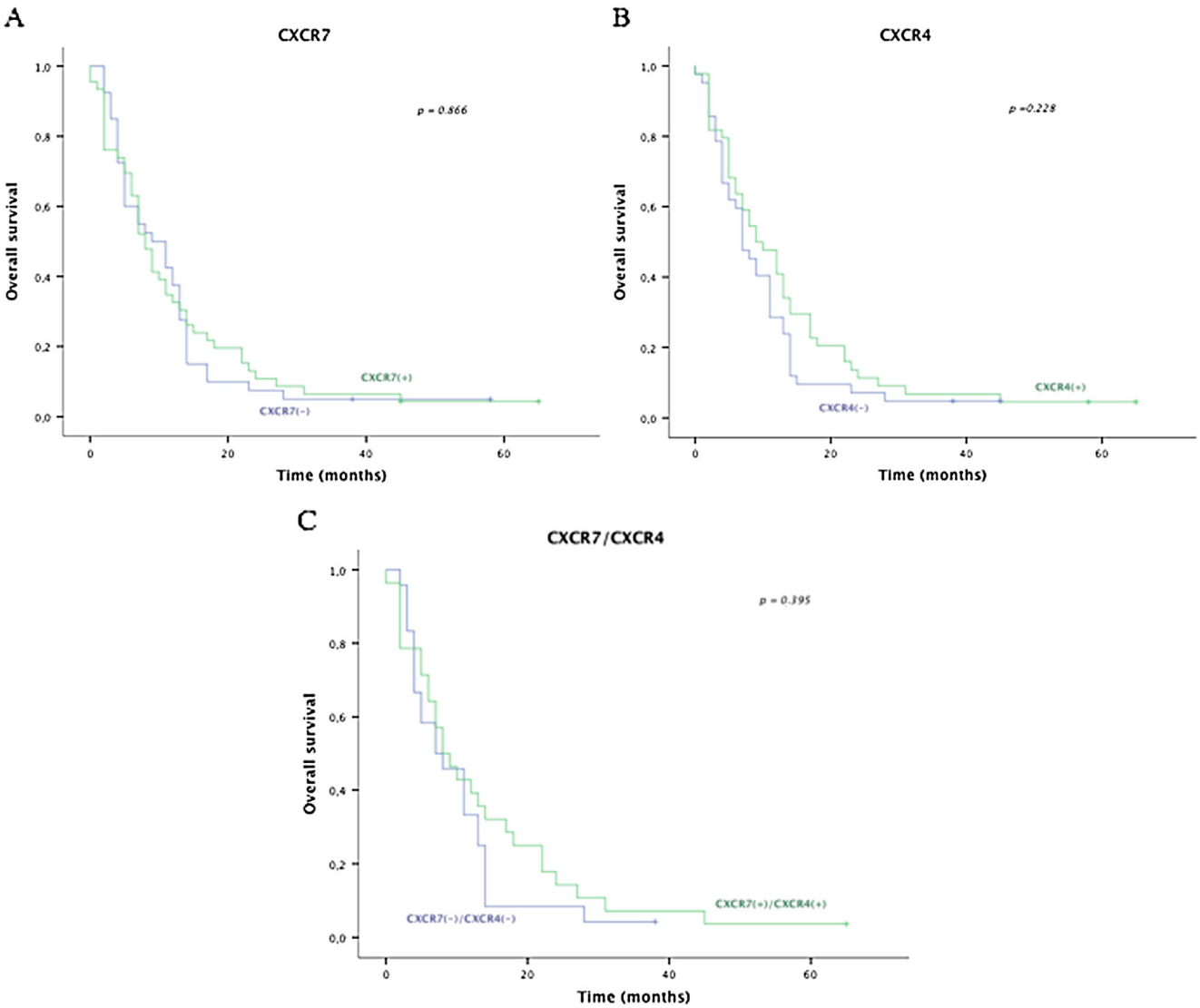

Fig. 8 Kaplan–Meier analysis samples of overall survival (OS) for patients with GBM. **a** $CXCR7^{+}$ vs $CXCR7^{-}$; **b** $CXCR4^{+}$ vs $CXCR4^{-}$; and, **c** $CXCR7^{+}/CXCR4^{+}$ vs $CXCR7^{-}/CXCR4^{-}$

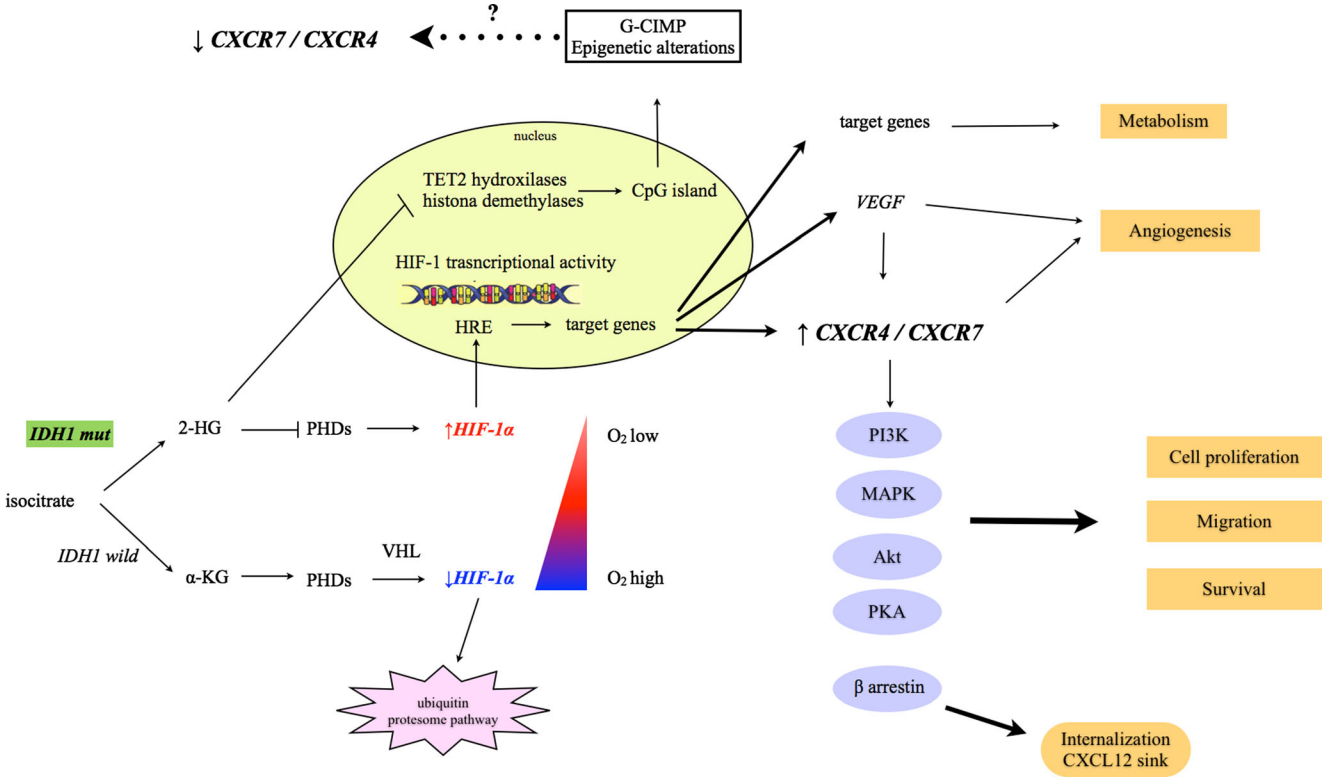

Fig. 9 Diagram illustrating possible mechanistic function of *CXCR7* and *CXCR4* expressions levels with *IDH1* mutation and *HIF1α* expression. IDHs enzymes normally catalyze the oxidative decarboxylation of isocitrate to α-KG witch plays important role in the degradation of HIF-1α. *IDH1* mutant exhibits a gain of function by catalyzing the production of 2-HG. High levels of this oncometabolite inhibit α-KG dependent dioxygenases. The inhibition of TET2 hydroxylases and histone demethylases probably leads to increased DNA hypermethylation. This induces a methilator phenotype that could exert an epigenomic inhibtory effect on *CXCR7/CXCR4*. The 2-HG also influences HIF in some conditions (hypoxic), inhibition of prolyl hydroxylases (PHDs) can lead to increased levels of HIF downstream. Binding to the hypoxia response element (HRE), inducing the transcriptional activity of genes. Biological effects of CXCR4/CXCR7 are mediated by activation of GPCR. Upon ligand binding activates PI3K, MAPK, Akt, PKA pathways

CXCR7/HIF1a was reported. Association with *HIF1a* was significant only in GBM.

These findings allow us to speculate that hypoxia, more relevant in GBM, activates the expression of *HIF1α* and this modulates the expression of *CXCR4* and *CXCR7*, both related to the process of angiogenesis, marked a change in GBM. Increased expression of *CXCR7* in tumor endothelium GBM, demonstrated in this study, should contribute to this process of angiogenesis.

It has been well documented that activation of the HIF1 pathway may induce local expression of angiogenic factors, including CXCL12.[47–49] Low oxygen concentration induces high expression of the *CXCR4* in different cell types and increases the recruitment of multiple populations of cells derived from the bone marrow.[23, 50, 51] Moreover, *CXCR4* and *CXCR7* mRNA expressions have been demonstrated to be up-regulated by hypoxia in human microvascular endothelial cells.[52] Knockdown of *HIF1α* in glioma cells significantly impaired the migration of tumor cells *in vitro* and their ability to invade the brain parenchyma *in vivo*.[53] Our results of *HIF1α* overexpression related to higher *CXCR7* and *CXCR4* expressions corroborate these previously reported data.

IDH1 mutation correlate strongly with the grade of astrocytoma. *IDH1* mutation was found in 84 % of AGII, 50 % of AGIII, and 10 % of GBM in our series. *IDH1mut* related to a significantly lower expression of both receptors, *CXCR4* and *CXCR7*, but presented no significant impact on the expression of *HIF1α* corroborating previous observations by others.[54][55] In fact, it have been demonstrated that 2HG, the oncometabolite generated by *IDH1* mutation, inhibits α-KG-dependent dioxygenases. These enzymes modulate several pathways, including sensitization to hypoxia, demethylation of histones and DNA, fatty acid metabolism and modifications of collagen, contributing to tumorigenesis. Furthermore, the presence of *IDH1* mutation has been tightly associated to CpG island methylator phenotype[56], and more recently it was shown that *IDH1* mutation only is sufficient to establish the glioma hypermethylator phenotype[57]. The induction of a methylated phenotype provides the molecular basis for the therapeutic response to alkylating agent as temozolomide[58]. Therefore, the double hit of presence of *IDH1* mutation and *MGMT* gene methylation lead to a favorable impact on overall survival among low-grade gliomas.[59]

The presence of *IDH1* mutation modulates to a minor increase in these expressions of *CXCR4* and *CXCR7*, possibly by epigenetic phenomena, less stimulus for angiogenesis and in this context may explain the better prognosis of patients with this type of mutation. Figure 9.

Conclusion

This study showed that *CXCR7* gene differentially expressed in astrocytomas, protein expression levels correlate with the degree of malignancy. Overexpression *HIF1α* relates with higher expressions of *CXCR7* and *CXCR4*, otherwise *IDH1mut* relates with lower expressions.

CXCR7 might play an additional role in the tumorigenesis of astrocytomas and further studies to elucidate its mechanistic function would be worthwhile.

Acknowledgments The authors state that neither the submitted paper nor any similar paper, in whole or in part, other than an abstract or preliminary communication, has been or will be submitted to or published in any other source.

Conflict of Interest There is no conflict of interest to declare.

Funding This work was supported by FAPESP projets: # 01/12898-4 and 04/12133-6.

References

1. Dolecek TA, Propp JM, Stroup NE, Kruchko C (2012) CBTRUS statistical report: primary brain and central nervous system tumors diagnosed in the United States in 2005–2009. Neuro Oncol 14(Suppl 5):v1–v49. doi:10.1093/neuonc/nos218
2. Kleihues P, Louis DN, Scheithauer BW et al (2002) The WHO classification of tumors of the nervous system. J Neuropathol Exp Neurol 61:215–225, discussion 226–229
3. Janny P, Cure H, Mohr M et al (1994) Low grade supratentorial astrocytomas. Manag Prognostic Factors Cancer 73:1937–1945
4. Keles GE, Lamborn KR, Berger MS (2001) Low-grade hemispheric gliomas in adults: a critical review of extent of resection as a factor influencing outcome. J Neurosurg 95:735–745. doi:10.3171/jns.2001.95.5.0735
5. Leighton C, Fisher B, Bauman G et al (1997) Supratentorial low-grade glioma in adults: an analysis of prognostic factors and timing of radiation. J Clin Oncol 15:1294–1301
6. Schomas DA, Laack NNI, Rao RD et al (2009) Intracranial low-grade gliomas in adults: 30-year experience with long-term follow-up at Mayo clinic. Neuro Oncol 11:437–445. doi:10.1215/15228517-2008-102
7. Chang EF, Smith JS, Chang SM et al (2008) Preoperative prognostic classification system for hemispheric low-grade gliomas in adults. J Neurosurg 109:817–824. doi:10.3171/JNS/2008/109/11/0817
8. Van den Bent MJ, Afra D, de Witte O et al (2005) Long-term efficacy of early versus delayed radiotherapy for low-grade astrocytoma and oligodendroglioma in adults: the EORTC 22845 randomised trial. Lancet 366:985–990. doi:10.1016/S0140-6736(05)67070-5
9. Stupp R, Mason WP, van den Bent MJ et al (2005) Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. N Engl J Med 352:987–996. doi:10.1056/NEJMoa043330
10. Balkwill F (2004) Cancer and the chemokine network. Nat Rev Cancer 4:540–550. doi:10.1038/nrc1388
11. Gerber PA, Hippe A, Buhren BA et al (2009) Chemokines in tumor-associated angiogenesis. Biol Chem 390:1213–1223. doi:10.1515/BC.2009.144
12. Kakinuma T, Hwang ST (2006) Chemokines, chemokine receptors, and cancer metastasis. J Leukoc Biol 79:639–651. doi:10.1189/jlb.1105633
13. Calatozzolo C, Canazza A, Pollo B et al (2011) Expression of the new CXCL12 receptor, CXCR7, in gliomas. Cancer Biol Ther 11:242–253
14. D'Alterio C, Consales C, Polimeno M et al (2010) Concomitant CXCR4 and CXCR7 expression predicts poor prognosis in renal cancer. Curr Cancer Drug Targets 10:772–781
15. Gebauer F, Tachezy M, Effenberger K et al (2011) Prognostic impact of CXCR4 and CXCR7 expression in pancreatic adenocarcinoma. J Surg Oncol 104:140–145. doi:10.1002/jso.21957
16. Hattermann K, Held-Feindt J, Lucius R et al (2010) The chemokine receptor CXCR7 is highly expressed in human glioma cells and mediates antiapoptotic effects. Cancer Res 70:3299–3308. doi:10.1158/0008-5472.CAN-09-3642
17. Balabanian K, Lagane B, Infantino S et al (2005) The chemokine SDF-1/CXCL12 binds to and signals through the orphan receptor RDC1 in T lymphocytes. J Biol Chem 280:35760–35766. doi:10.1074/jbc.M508234200
18. Burns JM, Summers BC, Wang Y et al (2006) A novel chemokine receptor for SDF-1 and I-TAC involved in cell survival, cell adhesion, and tumor development. J Exp Med 203:2201–2213. doi:10.1084/jem.20052144
19. Arvidsson Y, Bergström A, Arvidsson L et al (2010) Hypoxia stimulates CXCR4 signalling in ileal carcinoids. Endocr Relat Cancer 17:303–316. doi:10.1677/ERC-09-0085
20. Liu H, Xue W, Ge G et al (2010) Hypoxic preconditioning advances CXCR4 and CXCR7 expression by activating HIF-1α in MSCs. Biochem Biophys Res Commun 401:509–515. doi:10.1016/j.bbrc.2010.09.076
21. Liu Y-L, Yu J-M, Song X-R et al (2006) Regulation of the chemokine receptor CXCR4 and metastasis by hypoxia-inducible factor in non small cell lung cancer cell lines. Cancer Biol Ther 5:1320–1326
22. Sun X, Wei L, Chen Q, Terek RM (2010) CXCR4/SDF1 mediate hypoxia induced chondrosarcoma cell invasion through ERK signaling and increased MMP1 expression. Mol Cancer 9:17. doi:10.1186/1476-4598-9-17
23. Schioppa T, Uranchimeg B, Saccani A et al (2003) Regulation of the chemokine receptor CXCR4 by hypoxia. J Exp Med 198:1391–1402. doi:10.1084/jem.20030267
24. Parsons DW, Jones S, Zhang X et al (2008) An integrated genomic analysis of human glioblastoma multiforme. Science 321:1807–1812. doi:10.1126/science.1164382
25. Dang L, White DW, Gross S et al (2009) Cancer-associated IDH1 mutations produce 2-hydroxyglutarate. Nature 462:739–744. doi:10.1038/nature08617
26. Göttle P, Kremer D, Jander S et al (2010) Activation of CXCR7 receptor promotes oligodendroglial cell maturation. Ann Neurol 68:915–924. doi:10.1002/ana.22214
27. Valente V, Teixeira SA, Neder L et al (2009) Selection of suitable housekeeping genes for expression analysis in glioblastoma using quantitative RT-PCR. BMC Mol Biol 10:17. doi:10.1186/1471-2199-10-17
28. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)) Method. Methods 25:402–408. doi:10.1006/meth.2001.1262

29. Uno M, Oba-Shinjo SM, da Silva R et al (2011) IDH1 mutations in a Brazilian series of Glioblastoma. Clinics (Sao Paulo) 66:163–165
30. Hermann PC, Huber SL, Herrler T et al (2007) Distinct populations of cancer stem cells determine tumor growth and metastatic activity in human pancreatic cancer. Cell Stem Cell 1:313–323. doi:10.1016/j.stem.2007.06.002
31. Ehtesham M, Mapara KY, Stevenson CB, Thompson RC (2009) CXCR4 mediates the proliferation of glioblastoma progenitor cells. Cancer Lett 274:305–312. doi:10.1016/j.canlet.2008.09.034
32. Salmaggi A, Maderna E, Calatozzolo C et al (2009) CXCL12, CXCR4 and CXCR7 expression in brain metastases. Cancer Biol Ther 8:1608–1614
33. Hartmann TN, Burger M, Burger JA (2004) The role of adhesion molecules and chemokine receptor CXCR4 (CD184) in small cell lung cancer. J Biol Regul Homeost Agents 18:126–130
34. Wang J, Shiozawa Y, Wang J et al (2008) The role of CXCR7/RDC1 as a chemokine receptor for CXCL12/SDF-1 in prostate cancer. J Biol Chem 283:4283–4294. doi:10.1074/jbc.M707465200
35. Miao Z, Luker KE, Summers BC et al (2007) CXCR7 (RDC1) promotes breast and lung tumor growth in vivo and is expressed on tumor-associated vasculature. Proc Natl Acad Sci U S A 104:15735–15740. doi:10.1073/pnas.0610444104
36. Iwakiri S, Mino N, Takahashi T et al (2009) Higher expression of chemokine receptor CXCR7 is linked to early and metastatic recurrence in pathological stage I nonsmall cell lung cancer. Cancer 115:2580–2593. doi:10.1002/cncr.24281
37. Zheng K, Li H-Y, Su X-L et al (2010) Chemokine receptor CXCR7 regulates the invasion, angiogenesis and tumor growth of human hepatocellular carcinoma cells. J Exp Clin Cancer Res 29:31. doi:10.1186/1756-9966-29-31
38. Liang JJ, Zhu S, Bruggeman R et al (2010) High levels of expression of human stromal cell-derived factor-1 are associated with worse prognosis in patients with stage II pancreatic ductal adenocarcinoma. Cancer Epidemiol Biomarkers Prev 19:2598–2604. doi:10.1158/1055-9965.EPI-10-0405
39. Wang L, Chen W, Gao L et al (2012) High expression of CXCR4, CXCR7 and SDF-1 predicts poor survival in renal cell carcinoma. World J Surg Oncol 10:212. doi:10.1186/1477-7819-10-212
40. Hao M, Zheng J, Hou K et al (2012) Role of chemokine receptor CXCR7 in bladder cancer progression. Biochem Pharmacol 84:204–214. doi:10.1016/j.bcp.2012.04.007
41. Maishi N, Ohga N, Hida Y et al (2012) CXCR7: a novel tumor endothelial marker in renal cell carcinoma. Pathol Int 62:309–317. doi:10.1111/j.1440-1827.2012.02792.x
42. Schrevel M, Karim R, ter Haar NT et al (2012) CXCR7 expression is associated with disease-free and disease-specific survival in cervical cancer patients. Br J Cancer 106:1520–1525. doi:10.1038/bjc.2012.110
43. Takano S, Yamashita T, Ohneda O (2010) Molecular therapeutic targets for glioma angiogenesis. J Oncol 2010:351908. doi:10.1155/2010/351908
44. Wang Y-N, Hung M-C (2012) Nuclear functions and subcellular trafficking mechanisms of the epidermal growth factor receptor family. Cell Biosci 2:13. doi:10.1186/2045-3701-2-13
45. Brand TM, Iida M, Li C, Wheeler DL (2011) The nuclear epidermal growth factor receptor signaling network and its role in cancer. Discov Med 12:419–432
46. Wang S-C, Hung M-C (2009) Nuclear translocation of the epidermal growth factor receptor family membrane tyrosine kinase receptors. Clin Cancer Res 15:6484–6489. doi:10.1158/1078-0432.CCR-08-2813
47. Rong Y, Durden DL, Van Meir EG, Brat DJ (2006) "Pseudopalisading" necrosis in glioblastoma: a familiar morphologic feature that links vascular pathology, hypoxia, and angiogenesis. J Neuropathol Exp Neurol 65:529–539
48. Kioi M, Vogel H, Schultz G et al (2010) Inhibition of vasculogenesis, but not angiogenesis, prevents the recurrence of glioblastoma after irradiation in mice. J Clin Invest 120:694–705. doi:10.1172/JCI40283
49. Ceradini DJ, Kulkarni AR, Callaghan MJ et al (2004) Progenitor cell trafficking is regulated by hypoxic gradients through HIF-1 induction of SDF-1. Nat Med 10:858–864. doi:10.1038/nm1075
50. Pugh CW, Ratcliffe PJ (2003) Regulation of angiogenesis by hypoxia: role of the HIF system. Nat Med 9:677–684. doi:10.1038/nm0603-677
51. Zagzag D, Lukyanov Y, Lan L et al (2006) Hypoxia-inducible factor 1 and VEGF upregulate CXCR4 in glioblastoma: implications for angiogenesis and glioma cell invasion. Lab Invest 86:1221–1232. doi:10.1038/labinvest.3700482
52. Schutyser E, Su Y, Yu Y et al (2007) Hypoxia enhances CXCR4 expression in human microvascular endothelial cells and human melanoma cells. Eur Cytokine Netw 18:59–70. doi:10.1684/ecn.2007.0087
53. Mendez O, Zavadil J, Esencay M et al (2010) Knock down of HIF-1? in glioma cells reduces migration in vitro and invasion in vivo and impairs their ability to form tumor spheres. Mol Cancer 9:133. doi:10.1186/1476-4598-9-133
54. Metellus P, Colin C, Taieb D et al (2011) IDH mutation status impact on in vivo hypoxia biomarkers expression: new insights from a clinical, nuclear imaging and immunohistochemical study in 33 glioma patients. J Neurooncol 105:591–600. doi:10.1007/s11060-011-0625-2
55. Williams SC, Karajannis MA, Chiriboga L et al (2011) R132H-mutation of isocitrate dehydrogenase-1 is not sufficient for HIF-1α upregulation in adult glioma. Acta Neuropathol 121:279–281. doi:10.1007/s00401-010-0790-y
56. Noushmehr H, Weisenberger DJ, Diefes K et al (2010) Identification of a CpG island methylator phenotype that defines a distinct subgroup of glioma. Cancer Cell 17:510–522. doi:10.1016/j.ccr.2010.03.017
57. Turcan S, Rohle D, Goenka A et al (2012) IDH1 mutation is sufficient to establish the glioma hypermethylator phenotype. Nature 483:479–483. doi:10.1038/nature10866
58. Hegi ME, Diserens A-C, Gorlia T et al (2005) MGMT gene silencing and benefit from temozolomide in glioblastoma. N Engl J Med 352:997–1003. doi:10.1056/NEJMoa043331
59. Leu S, von Felten S, Frank S et al (2013) IDH/MGMT-driven molecular classification of low-grade glioma is a strong predictor for long-term survival. Neuro Oncol 15:469–479. doi:10.1093/neuonc/nos317