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Hypoxia-associated factor expression in low-grade and anaplastic gliomas: a marker of poor outcome

Aurélie Tchoghandjian¹,², Mei Y. Koh³, David Taieb¹,²,⁴, Sara Ganaha⁶, Garth Powis⁵, Emilie Bialecki⁵, Noël Graziani⁵, Dominique Figarella-Branger¹,²,⁶, Philippe Metellus²,⁵

¹Aix-Marseille Université, Faculté de Médecine, Marseille, 13385 Cedex 05, France
²INSERM, UMR 911, CRO2, Faculté de Médecine, Marseille, 13385 Cedex 05, France
³Sanford-Burham Medical Research Institute, Basic Laboratory Cancer Center, La Jolla, 92037 California, USA
⁴Department of Nuclear Medicine, Assistance Publique-Hôpitaux de Marseille, Hôpital de la Timone, Marseille, 13385 Cedex 05, France
⁵Department of Neurosurgery, Centre Hospitalier Clairval, Ramsay Generale de Sante, Marseille, 13009, France
⁶Department of Neuropathology, Assistance Publique-Hôpitaux de Marseille, Hôpital de la Timone, Marseille, 13385 Cedex 05, France

Correspondence to: Philippe Metellus, e-mail: Philippe.metellus@outlook.fr

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ABSTRACT

Somatic mutations in isocitrate dehydrogenase (IDH) genes have recently been identified in a large proportion of glial tumors of the CNS and reported to be a strong prognostic factor in gliomas whatever the tumor grade. Few data are available in the literature regarding the relationship between IDH mutations and HIF expression in low-grade gliomas (LGGs), especially in a recently described aggressive molecular subtype: “triple negative” (IDH non mutated, 1p 19q non codeleted, p53 expression negative) gliomas. We analyzed clinical, radiological and molecular features of a series of 31 grade II/III gliomas. p53 expression, 1p19q deletion and IDH mutation status were provided for all tumors. Also HIF (hypoxia inducible factor)-1α, HIF-2α, HAF, Sox2 (SRY(Sex determining region Y)-box2) and OCT (octamer binding factor) 3/4 expressions were analyzed. We found positive HIF-2α staining in 38.7% of cases which was uncorrelated to HIF-1α expression or IDH1/2 mutation status. However, HIF-2α staining was significantly associated with HAF expression, a stem-like cell marker, in the whole population. HAF expression was present in 74.2% of cases and significantly correlated to Sox2 expression. Furthermore, HAF expression was significantly associated with the “triple negative” glioma phenotype. We provide here first evidence that HAF, a stem-like cell marker, expression is highly correlated to the triple negative aggressive LGG/AG molecular phenotype suggesting that these tumours might arise from cells of different origin.

INTRODUCTION

Somatic mutations in isocitrate dehydrogenase enzyme isoforms 1 (IDH1) and 2 (IDH2) genes (IDH1/2) have recently been identified in a large proportion of glial tumors of the CNS (Central nervous System) [1-4]. IDH1 mutation has been reported to be a strong and independent indicator for good prognosis in gliomas whatever the tumor grade [5-10]. The biochemical consequences of IDH1 mutations are complex and remain controversial. It was initially reported that IDH1 mutations led to HIF-1 stabilization, a finding which was attributed to an inhibition of PHDs (propyl hydroxylases) by 2-HG (2-hydroxy-glutarate), a widely accepted mechanism for the succinate and fumarate SDH (succinate dehydrogenase) or FH (fumarate hydroxylase)-related tumors, respectively. However,
there are conflicting results regarding the role of HIF-1 in IDH1/2-related tumors. Koivunen et al reported that 2-HG efficiently inhibits a number of 2-OG-dependent dioxygenases, but does not inhibit PHDs. Instead, 2-HG was capable of promoting the activity of PHD1 and 2, and to a lesser extent, the activity of PHD3, leading to the downregulation of the amount of HIF-1α [11]. Taken together, the findings challenge the current HIF-hypothesis in IDH1/2-mutated tumors.

We recently showed that IDH1/2-related low-grade and anaplastic gliomas were not associated with an activation of HIF-1α [12] and HIF-1α overexpression was restricted to necrotic areas. These results are consistent with the findings of Williams et al [13]. However, it has recently become clear that HIF-1α, and its closely-related isoform, HIF-2α, act in concert in regulating the response to hypoxia, exhibiting unique functions such as the regulation of the glycolytic enzymes and PDK1 by HIF-1α, and erythropoiesis and cellular iron metabolism by HIF-2α, while also sharing some redundancies such as in the regulation of angiogenesis, lipid metabolism, and metastasis [14]. A recent study showed that chronic hypoxic or pseudo-hypoxic conditions were associated with a switch from HIF1-alpha to HIF-2α [15], a finding which seems to occur naturally as well as during tumorigenesis. The hypoxia-associated factor (HAF) is an isoform specific E3 ubiquitin ligase that specifically degrades HIF-1α but not HIF-2α, and promotes HIF-2α transactivation, thus promoting the switch from HIF-1 to HIF-2 dependent transcription [16-18]. The consequences of the shift towards HIF-2 dependent transcription include a shift towards an undifferentiated phenotype, which is typically associated with more aggressive disease. Until the present, no data exists regarding the expression of HIF-2α in gliomas.

Therefore, we have evaluated the expression of HAF, HIF-2α and HIF-2α targets genes (Sox2, OCT3/4) in a cohort of low grade gliomas (LGGs) and anaplastic gliomas (AGs).

RESULTS

According to the WHO classification, there were 23 (74.2%) grade II and 8 (25.8%) grade III gliomas. All tumors were supratentorial. Histologically there were 4 (12.9%) astrocytomas, 12 (38.7%) oligoastrocytomas and 15 (48.4%) pure oligodendrogliomas. Necrosis was absent in all cases. WHO grade or histological type were not significantly associated with IDH status. 1p19q deletion was found in 10/18 (55.5%, p=0.001) of IDH mutated tumors and p53 expression in 9/18 (50%; p=0.003) of these IDH mutated tumors.

All 31 tumors were analyzed for the presence of codons 132 and 172 mutations on the IDH1 and IDH2 genes respectively. IDH mutations were found in 18/31 (60.6%) tumors. Sequence analysis of exon 4 of IDH1 gene revealed mutations in 17 out of 31 (54.8%) patients at residue R132, including 15/17 (88.2) R132H (p.Arg132His, c.395G>A), 1/17 (5.6%) R132C (p.Arg132Cys, c.394C>T) and 1/17 (5.6%) R132S (p.Arg132Ser, c.394C>A). Sequence analysis of exon 4 of the IDH2 gene revealed 1/31 mutation (5.8%) at residue R172M (p.Arg172Met, c.515G>T). This patient harbored pure oligodendroglioma as reported by others [19].

Sex ratio was 1.38 (18 male and 13 female), and median age was 40 years (range, 22-71 years). Triple negative (IDH non mutated, 1p 19q non codeleted, p53 expression negative) tumor patients presented with a significantly higher age (55.5yrs vs 37.5yrs, p=0.002). The characteristics of this population are listed in Table 1.

HAF, HIF-2α, HIF-1α, Sox2 and OCT3/4 expression

We found that HAF expression was present in 74.2% of cases and was significantly correlated to Sox2 expression, a stem-cell marker (p=0.02). Furthermore, HAF expression was significantly associated (p=0.0004) with the “triple negative” glioma phenotype, we previously described, that combines IDH-non mutated, p53 non mutated and 1p19q non deleted tumors (Figure 1, Table 1) [7, 8]. Triple negative gliomas share peculiar clinical characteristics, radiologic presentation, molecular features and dismal prognosis. Indeed, their median overall survival is significantly worse than their IDH mutated counterparts as reported by others [9, 10, 20]. Here, we first provide evidence that HAF expression is highly correlated to this aggressive glioma molecular phenotype.

Interestingly, we found positive HIF-2α staining in 38.7% of cases which was uncorrelated to HIF-1α expression or IDH1/2 mutation status. However, HIF-2α staining was significantly associated with HAF expression in the whole population (p=0.002). There was also a trend toward correlation between positive HIF-2α expression and HAF expression in IDH mutated tumors (p=0.08).

Finally OCT3/4 immunostaining was negative in all tumors.

Triple negative tumors were associated with a peculiar clinical features, radiologic presentation and dismal prognosis

A total of 13 patients harbored neither IDH1 nor IDH2 mutations. These tumors occurred in older patients (>40 year-old, P=0.002). All these 13 tumors (100%) displayed a marked (score=2) infiltrative pattern on MR imaging whereas only 8/18 (44.5%) of mutated ones had this infiltration score (P = 0.0004). Axial T2 largest diameter was significantly greater (10/13 vs 6/12) in non-mutated tumors (P = 0.02). Greater extent of surgery (GTR and STR) was significantly lower in this group (2/13 vs 12/18, P<0.003).
Also temporo-insular location was significantly higher in this group (1/13 vs 13/18, \( P =0.0007 \)). Necrosis was absent in all cases (Table 2). A typical case of this clinical, radiologic and molecular subgroup is shown in Figure 1 (first column) along with a well-delineated fronto-temporo-insular IDH-mutated tumor Figure 1 (second column).

<table>
<thead>
<tr>
<th>Variables</th>
<th>All patients (n=31)</th>
<th>IDH mutated gliomas (n=18)</th>
<th>Triple negative gliomas (n=13)</th>
<th>P-value</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Number of pts (%)</td>
<td>Number of pts (%)</td>
<td>Number of pts (%)</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Median</td>
<td>40 yrs</td>
<td>37.5 yrs</td>
<td>55.5 yrs</td>
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<td>Mean (+/-SD)</td>
<td>45.2 +/-14.2 yrs</td>
<td>37.8 +/- 12.2 yrs</td>
<td>54.4 +/- 12.4 yrs</td>
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<td>Range</td>
<td>22-71 yrs</td>
<td>22-63 yrs</td>
<td>27-71 yrs</td>
<td></td>
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<tr>
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<tr>
<td>Male</td>
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<td>10 (55.5%)</td>
<td>8 (61.5%)</td>
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<tr>
<td>Female</td>
<td>13 (39.4%)</td>
<td>8 (44.5%)</td>
<td>5 (38.5%)</td>
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<td>WHO II</td>
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<td>16 (88.9%)</td>
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<td>WHO III</td>
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<td>2 (11.1%)</td>
<td>6 (46.2%)</td>
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<td>7 (53.8%)</td>
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<td>Oligodendroglia</td>
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<td>11 (61.1%)</td>
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<td>13 (100%)</td>
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<td>8 (44.5%)</td>
<td>0 (0%)</td>
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<tr>
<td>IHC Sox2</td>
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<td></td>
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<td>7 (38.9%)</td>
<td>9 (69.2%)</td>
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<tr>
<td>No</td>
<td>15 (48.4%)</td>
<td>11 (61.1%)</td>
<td>4 (30.8%)</td>
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</table>

SD=standard deviation

Table 1: Patients and tumor characteristics
Figure 1: First column: Immunochemistry analysis of a triple negative LGG showing absence of OCT3/4 and HIF-2α immunostaining, and high level of HAF and Sox2 immunostaining. Axial T2-weighted sequences MRI-scan displayed a huge infiltrative glioma with a temporo-insular topography. Second column: Immunochemistry analysis of a non-triple negative LGG showing absence of OCT3/4 and HIF-2α immunostaining, and low level of HAF and Sox2 immunostaining. Axial T2-weighted sequences MRI-scan displayed a sharp border glioma with a fronto-insular topography.
Molecular profile predicts outcome of LGGs and AGs

We analyzed the prognostic impact of IDH1 and IDH2 mutations in our population. Triple negative patients had a median OS time of 36 months and a 5-year survival rate of 36%. Median OS time was not reached for patients with either IDH1 or IDH2 mutations and 5-year survival rate was 90.1% (P<0.001) (Figure 2). Patients with overexpression of HAF had a median OS time of 56 months and a 5-year survival rate of 47%. Median OS time was not reached for patients with no overexpression of HAF and 5-year survival rate was 80.2% (P<0.001) (Figure 3).

DISCUSSION

HAF expression has already been described as a stem-like cell marker in glioblastomas, involved in neoangiogenesis [21, 22]. Although LGGs share the same histological phenotype, the differences in biology and clinical prognosis of LGGs suggest that these tumors might arise from cells of different origin.

Multipotent neural stem cells (NSC) and progenitors derived from developing or adult central nervous system (CNS) can be isolated and propagated in culture in the presence of mitogens. This involves the generation of free-floating spherical clusters, termed “neurospheres” [23]. Neurospheres consist of both multipotent stem cells and more restricted progenitors at different stages of differentiation. In addition to the heterogeneity that occurs within neurospheres, there is evidence that neurospheres derived from different regions of the developing or adult brain display unique characteristics with regards to growth, differentiation and gene expression [24]. Previous studies have shown that primary human brain tumors especially glioblastoma (GBM) contain cells with NSC features suggesting that GBMs may occur after malignant transformation of NSCs or progenitors [25-29]. In contrast to IDH mutated LGGs, the “triple negative” subgroup presents a rapid malignant course with worse clinical outcome [8]. Thus it is likely that several types of brain tumor-initiating cells play a role in LGGs initiation. Furthermore, the peculiar radiological aspect and preferred temporo-insular location of these tumors are consistent with such a developmental hypothesis. The clinical behavior, response to therapy of these aggressive gliomas is challenging. Recently, several molecular studies on gliomas highlighted a potential complex ordered processes of multiple clonal selection and evolutionary event that support this developmental hypothesis [30-33].

The human insular cortex, is considered the developmentally most primitive lobe of the telencephalon [34]. Three architectonical areas have been described within the insular cortex, an anterior agranular area, a mid-dysgranular area and a posterior granular area [35]. Also a chemoarchitectonical organization of the insula parallels the structural cytoarchitectonical one [36]. Thus the embryological development of the human insula has led to a heterogenous cytochimeoarchitectonic and functional interface between a multimodal area (prefrontal cortex) and a primary area (sensorimotor and auditory cortex).
Figure 2: Comparison of Kaplan-Meier overall survival curves according to IDH status (log-rank test, $P<0.001$).

Figure 3: Comparison of Kaplan-Meier overall survival curves according to HAF expression level (log-rank test, $P<0.001$).
[37]. It can be hypothesized that this developmental heterogeneity could be subserved by a stem- or progenitor cells heterogeneous distribution locally and that gliomagenesis could result from a peculiar cross-talk between micro-environment and specific glial cells. Molecular heterogeneity of low-grade gliomas involving the insula would probably reflect this local structural and functional heterogeneity.

This study has actual limits since it is a retrospective analysis of a small cohort and we do know that no definitive conclusions could be drawn by this work. However, it raise some interesting and relevant question regarding the gliomagenesis.

Here, we provide first evidence that HAF expression, a stem-like cell marker, is significantly associated with a peculiar glioma molecular profile responsible for a worse outcome and is also correlated to Sox2 expression. Interestingly, Sox2, a transcription factor that serves key functions during embryonic development and is involved in cancer stem cell maintenance has been recently identified as useful independent prognostic factors in gliomas [38, 39]. Also, we demonstrated that HIF-2α expression was significantly associated with HAF expression in the whole population but not so clearly in the triple negative subgroup. Thus, whether undifferentiated and aggressive phenotype of triple negative gliomas is HIF-2α dependent or independent could not be asserted and need to be investigated further. However, our data suggest that HAF expression could identify a specific subgroup of LGG with malignant transformation potential that should be taken into account to better tailor monitoring and treatment strategies.

MATERIALS AND METHODS

Human tumor samples datas

La Timone University Hospital Center Pathology database was queried for all archival surgical specimens of World Health Organization (WHO) grade II and III gliomas between 2008 and 2012 and then crossed with the Imaging database for pretherapeutic MRI available data. A total of 31 of 332 newly diagnosed patients with grade II or grade III glioma were included in this retrospective study. All patients were operated at our institution (Assistance Publique-Hôpitaux de Marseille, Marseille, France). Tumor specimens were obtained according to a protocol approved by the local institutional review board, ethics committee and conducted according to national regulations. All the patients provided written informed consent. All analyzed brain tumors were subjected to review by a single neuropathologist (DFB). In this study, all patients were first time operated on without prior history of radiation or chemotherapy. For all patients, clinical, radiological data, and follow-up were collected as previously reported [8].

1p19q and p53 status analysis

All analysis were performed on 5 μm tissue sections of formalin-fixed paraffin-embedded tissues when tissue material was available (Bouin-fixed tissues were not used). Presence of 1p19q deletion was studied in all samples by fluorescent in situ hybridization (FISH) with locus-specific probes for 1p36 and 19q13 as previously described [40]. In order to detect TP53 protein overexpression (DO-7 clone, 1/200, Dako, Trappes, France) in all samples, an automated immunohistochemistry (IHC) analysis was performed with avidin-biotin-peroxydase complex on the BenchMark XT system (Ventana Medical Systems, Tucson, Ariz) with a Ventana kit that included 3,3-diaminobenzidine reagent.

Genomic DNA extraction

Areas of viable and representative tumor following review of all blocks were marked by a single neuropathologist (DFB). Then, for the 31 samples, tumor DNA was extracted from twenty 10 μm sections of formalin-fixed paraffin-embedded tissues using the QIAmp DNA minikit, as described by the manufacturer (Qiagen, Courtaboeuf, France). Spectrophotometric quantification was performed on a Nanodrop ND-100 (Labtech, Palaiseau, France) and all DNA samples were diluted to 25 ng/μl.

IDH1 codon 132 and IDH2 codon 172 sequence analysis

IDH1 and IDH2 alterations of the mutational hotspot codons R132 and R172 were assessed by PCR amplification and direct DNA sequencing. First, genomic regions spanning codons R132 and R172 were amplified by classic PCR on a MJ Research PCT-100 thermocycler. Briefly, PCR amplification was performed in a total volume of 20 μl, consisting of 1 μl of DNA solution (25 ng/μl), 1 μl of forward and reverse primers (5 μM), and 10 μl of 2X PCR Master Mix (Abgene, Courtaboeuf, France). Then, direct DNA sequencing for IDH1 and IDH2 mutations was performed by Beckman Coulter Genomics sequencing service (Takeley, United Kingdom) from purified PCR products using the same specific primers as previously described [12].

IDH1, HIF-1α, HIF-2α, HAF, Sox2, OCT3/4 immunohistochemistry

R132H mutated form of IDH1, Hypoxia Inducible Factor (HIF-1α and HIF-2α) HAF, Sox2 and OCT3/4 immunohistochemistry analyses were carried out on all glioma samples. After steam-heat-induced antigen retrieval, 5 micrometer serial sections of formalin-fixed paraffin-embedded samples were tested for the presence of IDH1 R132H (mouse monoclonal antibody, H09 clone, Dianova, Germany), HIF-1α (rabbit polyclonal...
Statistical analysis

To compare patients’ characteristics according to IDH status, we used Pearson’s chi-square test, Fisher’s exact test and U Mann-Whitney test. Values of less than 0.05 were considered significant for each statistical analysis. Survivals were estimated by using the Kaplan-Meier method and curves were compared by using the log-rank test. (PASW Statistics 17.0).

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest related to this article.

REFERENCES


