

Prognostic significance of co-overexpression of the *EGFR/IGFBP-2/HIF-2A* genes in astrocytomas

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Abstract Overexpression of the *EGFR*, *IGFBP-2* and *HIF-2A* genes has been observed in high-grade astrocytomas and these genes seem to be functionally related to one another. This study aimed to define the profile of their expressions, interactions and correlation with clinical features and prognostic significance in microdissected tumor samples from 84 patients with astrocytomas of different grades and from 6 white matter non-neoplastic brain tissue sample. *EGFR*, *IGFBP-2* and *HIF-2A* gene expression levels were analyzed by quantitative real-time PCR and differed significantly between grades I–IV astrocytic tumors ($P < 0.0001$, $P < 0.0001$ and $P: 0.0013$, respectively) when analyzed by the Kruskal–Wallis test. Grade I astrocytomas presented gene expression levels similar to those encountered in samples of microdissected

white matter of non-neoplastic brain tissue. Overexpression of the *EGFR*, *IGFBP-2* and *HIF-2A* genes was significantly associated with lower 2-year survival ($P: 0.009$, $P: 0.0002$ and $P: 0.008$, respectively). Co-overexpression of these genes was strongly associated with high-grade gliomas and lower survival in univariate ($P < 0.0001$) and multivariate ($P: 0.009$) analysis, suggesting that the co-expression of the *EGFR/IGFBP-2/HIF-2A* pathway genes may have a more important clinical and biological impact than the expression of each individual gene alone. These data support the existence of a common pathway involving these genes that could contribute to the design of new target treatments.

Keywords Astrocytoma · *EGFR* · *IGFBP-2* · *HIF-2A* · CNS tumor · Glioblastoma

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Introduction

Astrocytic tumors are the most frequent primary brain tumors affecting children and adults [1, 2]. They represent a heterogeneous group of neoplasias that may differ in their localization, clinical findings, proliferation rate, invasive potential, and tendency to progression [3]. According to the World Health Organization (WHO), on the basis of clinicopathological criteria, these tumors are classified into: pilocytic astrocytomas (WHO grade I), circumscribed, with slow growth and rarely undergoing malignant transformation, usually affecting children, and three groups of diffusely infiltrative astrocytomas comprising diffuse astrocytomas (grade II) usually showing a more benign behavior, with slow growth and generally affecting young adults,

anaplastic astrocytomas (grade III), infiltrative, with high proliferative potential and affecting adults, and glioblastomas (grade IV), with malignant behavior and a high mortality rate, which may be primary or may develop from grades II and III astrocytomas (secondary) [3–5]. There is strong evidence suggesting that these clinicopathological differences reflect the type of genetic alteration acquired during the process of transformation [3–5].

Astrocytic tumors have been clearly differentiated by the determination of the profile of gene expression using microarrays [1, 6–9]. Among the major genes differentially expressed in these tumors are the epidermal growth factor receptor (*EGFR*), the hypoxia-induced transcription factor 2- α (*HIF-2A*), and the insulin-like growth factor-binding protein 2 (*IGFBP-2*) genes, which could be involved in an *EGFR/FKBP12/HIF-2A* pathway [1].

Despite the great progress in the understanding of the biology of these tumors over the last decades, more effective therapies have not been obtained. The discovery of new pathways involved in the proliferation of these tumors may provide potential therapeutic targets.

In the present study we analyzed the profile of expression of these genes in grades I, II, III and IV astrocytic tumors by quantitative real-time PCR (RQ-PCR) and their association with survival.

Patients and methods

Study population

We analyzed 84 fresh-frozen microdissected tumor samples obtained from gross total surgical resection including 40 primary grade IV, 11 grade III, 14 grade II and 19 grade I astrocytomas from consecutive patients admitted for diagnosis and treatment to the participating institutions. Diagnoses were made according to WHO criteria [3]. Six samples of microdissected white matter obtained from patients with mesial temporal lobe epilepsy refractory to medical treatment were used as control. The study was approved by the Ethics Committee of each institution and was based on the Helsinki convention criteria. Patient mean age was 55.7 years for grade IV astrocytomas, 27.4 years for grade III astrocytomas, 36.1 years for grade II astrocytomas, and 9.3 years for grade I astrocytomas.

RNA extraction and cDNA synthesis

Total cellular RNA was extracted using TRIzol LS Reagent (Invitrogen, Carlsbad, CA, USA) and RNA was reverse

transcribed to single-stranded cDNA using M-MLV RT (Invitrogen), according to the manufacturer's protocol.

Quantitative real-time PCR (RQ-PCR)

The mRNA expression levels of *EGFR*, *IGFBP-2* and *HIF-2A* and an endogenous housekeeping gene coding for beta-glucuronidase (*GUSB*) as a reference were quantified using real-time PCR analysis with a Gene Amp® 5700 Sequence Detection System (PE Applied Biosystems, Foster City, CA, USA). Amplification of specific PCR products was detected using the SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. All primers employed were cDNA specific and were purchased from Invitrogen (São Paulo, SP, Brazil). Primer combinations were: (a) *EGFR*, 5'-tcgacgcatacagctcagacc-3' (forward primer); 5'-tttgggaacggactggtttatg-3' (reverse primer); (b) *IGFBP-2*, 5'-cctctactccctgcacatcc-3' (forward primer); *IGFBP-2*, 5'-agggtgtacaggccatgctt-3' (reverse primer); (c) *HIF-2A*, 5'-agcctccatctgccatcagtc-3' (forward primer), 5'-cttgcacatgctgacaccttg-3' (reverse primer) and (d) *GUSB* 5'-gaaaatatgtggttgagagctcatt-3' (forward primer) and 5'-ccgagtgaagatcccctttta-3' (reverse primer). Blank and standard controls (calibrators) were run in parallel to verify amplification efficiency within each experiment. Each run was completed with a melting curve analysis to confirm the specificity of amplification and the lack of primer dimers. For relative quantification of gene expression, standard curves were constructed for each gene by considering at least three points in triplicate of 10-fold serial dilution of cDNA in water, starting from 1:10 of a volume of undiluted cDNA transcribed from 1.0 μ g total RNA. The slopes of standard curves ranged from -3.17 to -3.87.

To normalize for differences in the amount of total cDNA added to each reaction, *GUSB* gene expression was used as an endogenous control. The normalized expression level of each gene was determined by dividing the expression level of each gene studied by the expression level of the *GUSB* gene in the same sample. A known quantity of the glioblastoma cell line U343 was used as a standard control. The normalized expression level of each sample was referred to the U343 normalized value for the same gene, which was arbitrarily assumed to be value 1 [10]. All reactions were made in duplicate.

Statistical analysis

Data were analyzed statistically using the GraphPad Prism software, version 3.0 (GraphPad Software, San

Diego, CA, USA) and SPSS Graduate Pack 8.0 software (SPSS Inc, Chicago, IL, USA). The distribution of gene expression among the four different histological types was compared by the Kruskal–Wallis test. The Dunn Multiple Comparison post test was performed to compare the differences between each astrocytoma group. Patients with values above the median were considered to present overexpression of the genes studied. Overall survival was analyzed by the Kaplan–Meier plots and by the log-rank test. Multivariate analysis by the Cox proportional model was applied to assure the independence of the prognostic factors detected in univariate analysis. The level of significance was set at $P < 0.05$ in all analyses.

Results

Overall 2 year survival was 12.2%, 70.7%, 92.9% and 100% for grades IV, III, II and I astrocytomas, respectively. Among patients with glioblastomas, those younger than 45 years were found to have significantly higher 12 month and 2 year survival rates compared to patients older than 45 years (67% vs. 31% and 41% vs. 3%, respectively) ($P: 0.05$).

The median and mean expression of genes *EGFR*, *IGFBP-2* and *HIF-2A* in astrocytomas and in brain tissue is shown in Table 1.

Patients with grade I astrocytomas presented *EGFR*, *HIF-2A* and *IGFBP-2* levels similar to those encountered in samples of microdissected white matter of non-neoplastic brain tissue. (Table 1 and Fig. 1).

The expression levels of the *EGFR*, *IGFBP-2* and *HIF-2A* genes showed a highly significant statistical difference among the various tumor subtypes when compared by the Kruskal–Wallis test ($P < 0.0001$, $P < 0.0001$ and $P: 0.0013$, respectively). Comparison of the different malignant grades by the Dunn multiple comparisons test showed that *EGFR* levels were lower in grade I astrocytomas than in grade II ($P < 0.001$), III

($P < 0.001$) and IV ($P < 0.001$) astrocytomas. It is interesting to note that there was no statistically significant difference in *EGFR* expression between grades II, III and IV astrocytomas ($P > 0.05$). The expression of gene *IGFBP-2* was significantly higher in glioblastomas than in grade III ($P < 0.001$), II ($P < 0.001$) and I ($P < 0.05$) astrocytomas. No significant differences in the expression of this gene were observed between grades III, II and I tumors ($P > 0.05$). The expression of gene *HIF-2A* differed significantly between pilocytic astrocytomas and glioblastomas ($P < 0.001$). The graphs regarding the expression of these genes in the gliomas of different grades and in non-neoplastic brain tissue are shown in Fig. 1.

As shown in Fig. 2, analysis of the association between histological grade and co-expression of these genes revealed a directly proportional association with more aggressive histological types (grades III and IV).

Gene expression values above the median were found to be significantly associated with lower 2-year survival: 64.6% versus 43.3% ($P: 0.009$) for gene *EGFR*, 66.1% versus 40.4% ($P: 0.008$) for *HIF-2A* and 71.3% versus 32.8% ($P: 0.0002$) for *IGFBP-2*. The overall 2-year survival was also associated with the co-expression of these genes. Patients who presented expression above the median for the 3 genes studied had an overall 2-year survival rate of 6%, versus 55% for the increased expression of 2 genes, 62% for the increased expression of 1 gene, and 83% for the absence of increased expression ($P < 0.0001$) (Fig. 3). In grade IV astrocytomas, even though the survival of patients with expression of genes *EGFR*, *IGFBP-2* and *HIF-2A* above the median was lower, the difference was not statistically significant ($P: 0.09$, $P: 0.73$ and $P: 0.15$, respectively). However, patients with co-overexpression of the 3 genes studied had a significantly lower 2-year survival ($P: 0.03$) (Fig. 3).

Multivariate analysis of all astrocytic tumors using the Cox proportional model showed that WHO classification, co-overexpression of the 3 genes and *EGFR*

Table 1 Median (minimum and maximum) and mean values of expression of genes *EGFR*, *IGFBP2* and *HIF-2A* in astrocytomas and in non-tumoral brain tissue (NT brain)

		Global Astro (n: 84)	Astro IV (n: 40)	Astro III (n: 11)	Astro II (n: 14)	Astro I (n: 19)	NT brain (n: 6)
<i>EGFR</i>	Median	1.386 (0.008–127.4)	2.369 (0.008–127.4)	3.469 (0.01–5.08)	1.632 (0.38–25.75)	0.1057 (0.008–1.51)	0.6223 (0.198–1.01)
	Mean	5.40	7.674	3.379	7.106	0.249	0.5916
<i>IGFBP-2</i>	Median	0.2011 (0.0007–9.33)	0.5907 (0.003–9.33)	0.02 (0.0007–0.2204)	0.0108 (0.0001–3.92)	0.0869 (0.0004–1.06)	0.015 (0.0018–0.693)
	Mean	0.6493	0.9443	0.045	0.2978	0.2627	0.125
<i>HIF-2A</i>	Median	8.1821 (0.20–49.04)	11.29 (1.316–49.04)	12.71 (3.16–32.92)	13.67 (1.71–30.63)	4.162 (0.53–15.35)	2.790 (0.20–10.03)
	Mean	12.1399	14.81	13.83	12.89	5.23	4.796

Fig. 1 Expression profile of genes *EGFR*, *IGFBP-2* and *HIF-2A* in grades I–IV astrocytomas and in non-neoplastic brain tissue. The full line corresponds to the median value for each group

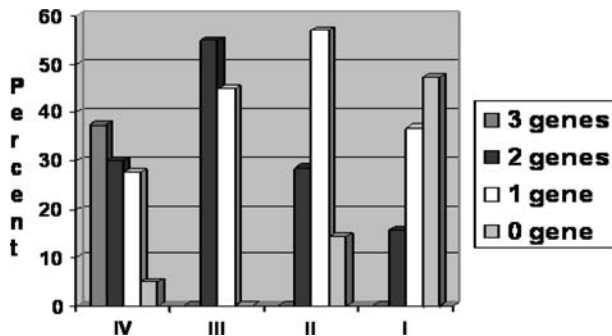
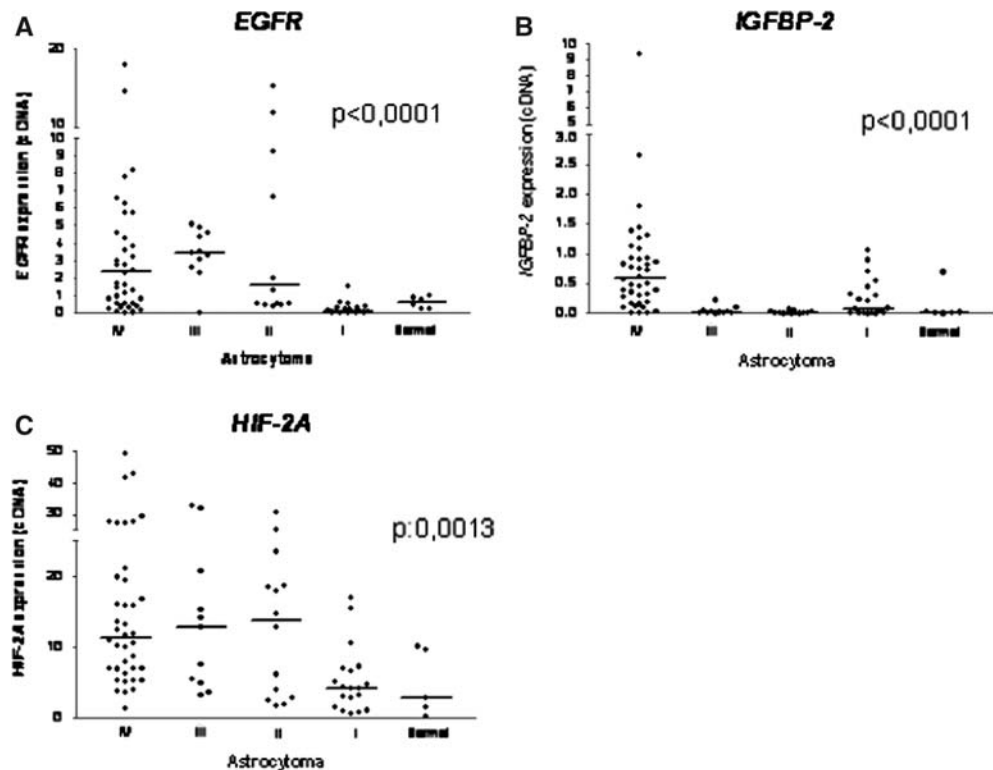


Fig. 2 Comparison of the co-overexpression for genes *EGFR*, *IGFBP-2* and *HIF-2A* and grades I–IV astrocytomas. (3 genes) represents the concomitant increased expression of the 3 genes, (2 genes) of 2 genes, (1 gene) of only 1 gene, and (0 gene) represents the absence of increased expression

overexpression were independent prognostic factors ($P < 0.0001$, $P: 0.009$ and $P: 0.05$ respectively). When only grade IV astrocytomas were analyzed, co-overexpression of the 3 genes was the most important independent prognostic factor, followed by age ($P: 0.02$ and $P: 0.03$ respectively).

Discussion

Isolated overexpression of the *EGFR*, *HIF-2A* and *IGFBP2* genes have been observed in high-grade

astrocytomas [1, 5, 10–14]. These genes seem to be functionally related to each other, acting as important promoters of angiogenesis and activation of pathways that result in enhancing of invasiveness, cellular differentiation, proliferation, migration and apoptosis inhibition in astrocytomas by activation of the P13K/Akt and Ras/MAPK pathways [1, 6, 7, 8, 15], suggesting that they could act in a common *EGFR*/*FKBP12*/*HIF-2A* pathway [1]. *HIF-2A* activation may be promoted by tyrosine-kinase receptors such as *EGFR* via activation of phosphatidylinositol 3'-kinase (PI3K)/protein kinase B (Akt) and multiple FBKPs (FK506 binding-proteins), especially FKBP12, resulting in activation of several target genes including *VEGF*, *PGK-1* and *IGFBP-2*, with a consequent increased angiogenesis, decreased apoptosis and tumor proliferation [1, 15].

In the present study, patients with grade I astrocytomas, a rich-vascular neoplasia [3], presented a gene expression profile similar to that encountered in samples of non-neoplastic brain tissue. This fact may explain in part the less infiltrative behavior of this tumor compared to astrocytomas grades II–IV. Although the analyzed genes are related to angiogenesis, their low expressions in grade 1 tumors suggest that other angiogenesis-related pathways could be involved in this tumor.

Regarding *EGFR* gene expression, it is interesting to emphasize that there was no statistically significant

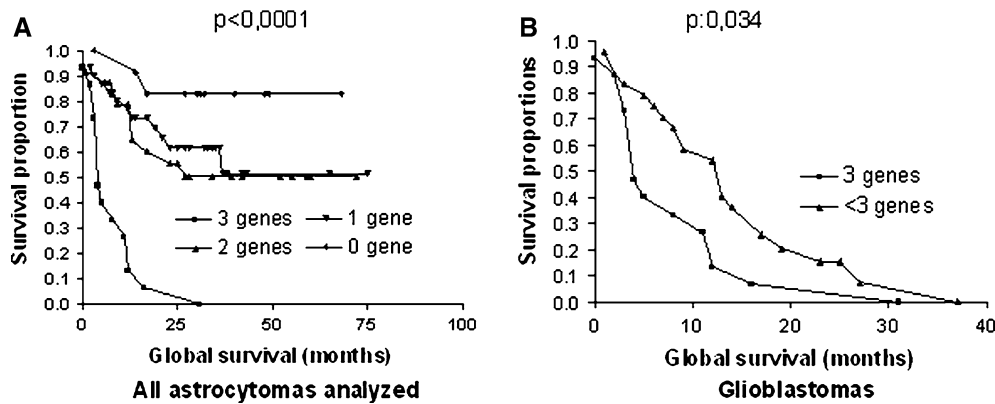


Fig. 3 (A) Global survival according to the increased co-expression of the genes under study. (3 genes) represents patients with expression above the median of the 3 genes, (2 gene) represents the increased expression of 2 genes, (1 gene)

the increased expression of only 1 gene, and (0 gene) the absence of increased expression of the 3 genes under study in all patients. **(B)** Global survival of patients with grade-IV glioblastomas with co-expression of the 3 genes versus <3 genes

difference between astrocytomas classified as diffusely infiltrative (WHO II-IV). *EGFR* expression in astrocytic tumors grade III and II has presented controversial. Some investigators have described *EGFR* amplification predominantly in high-grade astrocytomas (WHO III/IV) [11]. Other authors have recently reported *EGFR* overexpression in up to 28% of grade II astrocytomas [7, 12]. Grade II astrocytomas with overexpression of the *EGFR* gene also appear to have a more aggressive behavior [12]. Overexpression of *EGFR* gene has been frequently observed in cases with no amplification. The mechanism of over expression of *EGFR* has also been attributed to post-transcriptional activation, altered mRNA stability and changes in receptor degradation rates [11]. In our series in the grade II patients the *EGFR* expression level was lower than grade III and IV but with no statistically significance. In the present study, analysis of all astrocytoma subtypes showed that *EGFR* overexpression was significantly associated with a lower 2-year survival. For patients with glioblastomas, however, this association only tended to be significant ($P: 0.09$). This significantly higher expression in all infiltrative astrocytomas (II, III and IV) compared to pilocytic astrocytomas led us to assume that this gene may be associated, at least in part, with the more invasive phenotype not only in glioblastomas, but also in grades II and III astrocytomas compared to grade I astrocytomas. *EGFR* amplification has been associated with enhanced proliferation and a more aggressive behavior in glioblastomas [3]. In primary glioblastomas *EGFR* amplification or overexpression is associated with additional alteration on Rb pathway, specially overexpression of *MDM2* and inactivation of the *PI6* that could be the main pathway in the tumor development [3, 12].

Overexpression of *HIF-2A* has been described in prostate, renal, bladder, breast and head and neck cancers. It has been associated with aggressive biological behavior in these tumors and chemotherapy resistance, specially to platinum derivatives [16–20]. This gene is activated by hypoxia and also by growth factor and oncogenic pathways as IGF system, *VEGF*, mutant Ras and Scr pathways, mutations on suppressor tumor genes as *PTEN*, *TP53* and *PI4^{ARF}* [16–20]. Except for the data reported by Khatua et al. [1] for a small pediatric population with astrocytic tumors studied by microarray technology, to our knowledge, there are no reports regarding the expression of the *HIF-2A* gene in astrocytic tumors of different grades or its association with survival. The significantly lower expression in patients with grade I astrocytomas compared to patients with diffusely infiltrative tumors (grades II–IV) observed in the present study suggests that the expression of this gene may be associated with a higher invasive potential in astrocytic tumors. It is interesting to point out that its increased expression was also associated with a worse 2-year survival when all astrocytic tumors were analyzed as a whole. For grade IV astrocytomas, however, this association was not observed.

As also reported by others [2, 13], there was a significantly higher expression of *IGFBP-2* in grade IV than in grade I–III astrocytomas. *IGFBP-2* has been associated with invasiveness in grade IV astrocytomas and has presented close correlation with overexpression of *VEGF* [8] and the metalloproteinase *MMP-2*, an important regulator of the invasiveness and tumoral migration [2], reinforcing a more important activity of this gene in glioblastomas. Similar findings of greater expression of *IGFBP-2* in

high-grade tumors compared to low-grade tumors and non-neoplastic tissue have also been recently reported in breast tumors [21].

In the present study we frequently observed increased co-expression of these genes in several tumor samples, which strongly suggested a correlation among them in infiltrative gliomas, especially glioblastomas, when compared to pilocytic astrocytomas. This association also led to a significantly lower 2-year survival both for all astrocytomas analyzed and for glioblastomas, being a significant independent prognostic factor and suggesting for the first time that increased co-expression of these genes may have a more important clinical and biological impact than expression of each gene separately. This fact associated to reported data showing relationship among these genes [1, 5, 7, 15, 16, 18–20] support the hypothesis of a common pathway involving them and their association with more malignant behavior and shorter survival in gliomas.

Classifications based on patterns of gene expression in gliomas have shown a remarkable ability to distinguish ambiguous astrocytic tumors and have been associated with a better prognosis than that provided by histological classification, introducing the lack of the absolute reliability of the histological diagnosis of these tumors [6, 8, 9]. Our data reinforce the importance of classification based on gene expression patterns as complementary of the histological diagnosis that could provide an improvement in the stratification of the gliomas, specially glioblastomas.

These results may have direct therapeutic importance. New strategies of target therapy have been developed in oncology over the last few years, including target compounds against tyrosine-kinase receptors such as *EGFR* [22] and *FBPKs* [23]. The use of inhibitors of the HIF and IGFBP-2 systems has also been suggested as a possible therapeutic target [24, 25]. The use of combined target therapy in different synergic tumoral pathways including *EGFR* seems to present better results than the use of separate targets and had been used in clinical trials [25, 26]. We believe that the interaction observed between these genes could contribute to the design of new target treatment regimens for high-grade astrocytomas.

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