Prognostic value of mutations in isocitrate dehydrogenase 1 (IDH1) and reverse telomerase transcriptase (TERT) in Argentine patients’ gliomas

Valor pronóstico de mutaciones en Isocitrato dehidrogenasa 1 (IDH1) y Telomerasa transcriptasa reversa (TERT) en gliomas de pacientes argentinos

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Abstract

Background and aim: Gliomas are the most common primary brain tumors, classified according to their histopathological and genetic features. Tumorigenesis depends on alterations in different genes. The aim of this study was the identification of mutations in IDH1 and TERT genes in gliomas of Argentine patients and to correlate them with clinical features and prognosis. Methods: DNA was isolated from 19 biopsies with different glioma grades matched with blood samples. IDH1 and TERT mutations were studied by PCR amplification and sequencing. Results: Six out of seven patients with low-grade glioma (grade II) harbor IDH1 mutations, mainly without tumor growth and overall survival of more than 12 months. Eleven out of twelve patients with high-grade gliomas (grade III/IV) showed wild type IDH1, mainly with tumor growth and shorter survival than low-grade gliomas. Mutated TERT promoter was present in 5 out of 11 high-grade gliomas, showing the prevalence of polymorphic C allele. In 1 out of 5 low-grade gliomas with a predominance of T allele. IDH1 and TERT mutations were mutually exclusive in most gliomas. Conclusions: Our results show that genetic tests provided a more accurate prognosis than histopathological analysis. The evolution of gliomas can be predicted primarily by the mutational status of IDH1 and secondarily by other markers, such as TERT mutational status.

Keywords: gliomas; IDH1 mutations; TERT mutations; rs2853669; prognosis

Resumen

Antecedentes y objetivo: los gliomas son los tumores cerebrales primarios más comunes y se clasifican según sus características histopatológicas y genéticas. La tumorigénesis depende de alteraciones en diferentes genes. El objetivo de este estudio fue identificar mutaciones en los genes IDH1 y TERT en gliomas de pacientes argentinos y correlacionarlas con la evolución clínica. Métodos: se obtuvieron 19 muestras pareadas de ADN de gliomas y de la sangre. Las mutaciones en IDH1 y TERT se analizaron por PCR y secuenciación. Resultados: la IDH1 mutada se encontró en 6 de los 7 gliomas de bajo grado (grado II), mayormente sin crecimiento tumoral y una sobrevida mayor de 12 meses. La IDH1 salvaje estaba presente en 11 de los 12 gliomas de alto grado (grado III y IV) mayormente con crecimiento tumoral y menor sobrevida que los tumores de bajo grado. Las mutaciones en el promotor del gen TERT se observaron en 5 de los 11 gliomas de alto grado, con la prevalencia de alelo polymórfico C, en cambio, en gliomas de bajo grado TERT mutado estaba presente en 1 de los 5 gliomas con predominio del alelo T. Las mutaciones en IDH1 y TERT fueron mutuamente excluyentes en la mayoría de los gliomas. Conclusiones: el análisis genético provee un pronóstico más certero que el análisis histopatológico. Nuestros resultados muestran que la evolución de gliomas puede predecirse primariamente por el estado mutacional de IDH1 y secundariamente por mutaciones en otros marcadores tales como el TERT.

Palabras clave: gliomas; mutaciones en IDH1; mutaciones en TERT; rs2853669

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Running title: IDH1 and TERT mutations in gliomas

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Introduction

Gliomas are the most common primary brain tumors; they include clinically and genetically different kinds of neoplasms classified by the World Health Organization (WHO) according to their histopathological and genetic features, assigning them to grade I to IV (Louis et al., 2016). Grade II to IV are diffuse gliomas with an infiltrative characteristic that occur predominantly in adults. These tumors frequently localize in the cerebrum and preferentially in the frontal and temporal lobe. Clinical features are the consequences of distortion of the invaded anatomical structures. Prognosis of gliomas depends on histopathological characteristics and genetic alterations, mainly of the isocitrate dehydrogenase 1 (IDH1), 1p19q co-deletion, and methylation of MGMT promoter (Reuss et al., 2015; Louis et al., 2016). Thus, gliomas in adult patients classified according to histopathological characteristics and IDH1 mutational status are: 1) Grade II diffuse astrocytomas, IDH1 mutant; 2) Grade II diffuse astrocytomas, IDH1 wild type; 3) Grade II oligodendroglioma, IDH1 mutant, and 1p19q co-deletion; 4) Grade III anaplastic astrocytomas, IDH1 mutant; 5) Grade III anaplastic oligodendroglioma, IDH1 mutant, and 1p19q co-deletion; 6) Grade III anaplastic astrocytomas, sIDH1 wild type; 7) Grade IV glioblastoma IDH1 mutant; 8) Grade IV glioblastomas, IDH1 wild type, the most common and malignant astrocytic gliomas (Brennan et al., 2013). Patient’s age influences prognosis; younger patients have a better prognosis than elderly patients (65-70 years). Glioblastoma is highly resistant to current therapies with only modest survival (6 to 12 months).

Due to the high incidence and malignancy of gliomas, prediction of their evolution is essential, indicated by biological markers. IDH1/IDH2 mutation status is one of the validated markers for glioma prognosis. Mutations in IDH1 originate an oncometabolite (2-hydroxyglutarate) that causes hypermethylation in the promoter of different genes, silencing many differentiation factors and maintaining cells in a stem cell-like physiological state, prone to tumorigenesis. Another effect of mutant IDH1 is an alteration of the redox state and accumulation of free radicals leading to cellular damage. Both effects make the tumoral cells grow very slowly. (Lee et al. 2002; Parsons et al. 2008; Dang et al., 2009; Lu et al., 2012; Turcan et al., 2012). TERT promoter mutations C228T at the site -124bp and C250T at -146 bp result in the creation of consensus sequences TTCGG for ETC binding (E-twenty-six, ETS/TCF), which is associated with telomerase activation (Killela et al., 2013, Ramakrishna et al., 2015). A preexisting noncanonical ETS2 binding site (TTCCCA) disrupted by T>C transition leading to the variant allele of polymorphism rs2853669 at the site -245, which may affect the impact of TERT mutations on gliomas (Spiegler-Kreinecker et al., 2015; Batista et al., 2016).

In this work, we continue with the studies of molecular markers, previously carried out by others, providing more evidence on the value of the mutational status of IDH1, occurring mainly in low-grade gliomas, and TERT, a hallmark of high-grade gliomas, in a group of Argentine patients. Molecular results compared with clinical parameters: age, tumor grade, tumor growth, and overall-survival, determined their potential quality as prognostic markers.

Materials and methods

Ethic statements. This study approved by the Ethics Committee of “Facultad de Farmacia y Bioquímica” (Universidad de Buenos Aires, Argentina) and the Institutional Teaching and Research Committee of “Sanatorio Anchorena” (Buenos Aires, Argentina), where the surgeries performed. All patients gave their written informed consent. The study complied with all provisions of the Declaration of Helsinki.

Tumor samples. Glioma specimens obtained from series of patients studied in 2016. After magnetic resonance evaluation, only five patients underwent surgery, whereas the other 14 patients underwent stereotactic biopsy. High-quality snap-frozen tumor tissues, matched with blood samples, were obtained from 19 cases. The biopsies proceeded from 12 high-grade gliomas (III and IV) and seven low-grade gliomas (II).

Regarding therapy, ten patients with high-grade gliomas received a combined radio-chemotherapy with temozolomide: the remaining two patients died before the initiation of treatment. Only one patient with low-grade glioma received the same treatment due to a wild-type status of IDH1. Of the 19 patients, 10 were females with a mean age of 52 years and a range of 24-78 years, and 9 were males with a mean age of 46 years and 16-74 years.

Tumor growth was detected by Magnetic Resonance Imaging (MRI) during an observation period of 6 months and a 20% increase in the size considered a growth. Overall survival defined as the period between the date of surgery and death up to 18 months. A strictly (ambulatory) follow-up controlled the living patients. In Table 1, we summarize their clinical characteristics.

DNA extraction and mutation analysis of IDH1 and TERT. Tumor DNA prepared by treatment with proteinase K, purification with phenol/chloroform, and precipitation with ethanol. Blood DNA obtained by treating of leukocytes with CTAB (Cetyl hexadecyl Trimethyl Ammonium Bromide) purification with chloroform and precipitation with ethanol. The IDH1 region containing codon 132 was amplified using primers forward 5’ CAAATAGGGCGTCCATCTCAACACA 3’ and reverse 5’ TGCCACCAACGGACCAAGTC 3’ (Parson et al., 2008), resulting in a 481 bp PCR product. The TERT promoter region containing C228T and C250T mutations, as well as the polymorphism rs2853669, was amplified using primers forward 5’ GGCCGATTGCACTCTCTCTCTCT 3’ and reverse 5’ AGCACCCTGCAGGTAGTGGA 3’.
in the presence of DMSO 2.5% according to Killela et al. (2013), resulting in 489 bp fragments. PCR products were sequenced in the automated genetic-sequencer ABI 3730XL (Applied Biosystems, Macrogen) and analyzed by Finch TV program using as a reference the sequence of the IDH1 gene (Homo sapiens chromosome 2 GRCh38.p7 Primary Assembly. NCBI Reference Sequence NC_000002.12) and the TERT sequence (Homo sapiens chromosome 5, GRCh.p7, NCBI, NC_000005.10).

**Statistical Analyzes**

Statistical analyzes used the program GraphPad Prism 6, version 6.01 (GraphPad Prism Software, La Jolla, CA, USA). Association of IDH1 and TERT promoter mutations with clinicopathological parameters were determined, as they were qualitative variables, dichotomous and small in size (presence/absence of mutation, presence/absence of polymorphism, tumor growth yes-no, survival at 18 months yes-no), contingency tables performed using Fisher’s exact test and the Chi-Square with Yates correction. Kaplan-Meier survival analysis performed to estimate survival using a log-rank (Mantel-Cox). Assays with a p < 0.05 were considered statistically significant.

**Results**

Median age of patients was 49 years with a bimodal distribution in the third and sixth decade of life (Fig.1A). Patients with a high-grade glioma showed a decrease in survival compared to grade II glioma: 7 of the 12 patients with a high-grade glioma died in the postoperative period (6-10 month), whereas none of the seven patients with low glioma grade (II) died in this period (Table 1).

| Case | Age | Sex | Signs Symptoms | Localization | Surgical Treatment | WHO Grade | IDH1 gene | TERT gene | TERT SNP | Growth In 6 months | Overall survival |
|------|-----|-----|----------------|--------------|-------------------|------------|-----------|-----------|----------|-------------------|-----------------|
| 2    | 24  | Female | Seizures | Cortical Left Inferior Parietal (Wernicke) | SB | II | Mut | Wt | TT | Yes | Yes: (18 months of follow up) |
| 5    | 16  | Male | Disgraphy | Bithalamic | SB | II | Wt | Wt | TT | Yes | Yes: (8 months of follow up) |
| 8    | 37  | Female | Cephalea | Cortical Right Inferior Frontal | XS | II | Mut | ND | ND | No | Yes: (18 months of follow up) |
| 10   | 35  | Male | Seizures Mild Hemiparesis | Subcortical Right Rolandic | SB | II | Mut | ND | ND | No | Yes: (15 months of follow up) |
| 15   | 47  | Male | Mild Crural Paresis | Subcortical Left Rolandic | SB | II | Mut | Wt | TT | No | Yes: (7 months of follow up) |
| 12   | 32  | Male | Seizures | Corpus Callosum Base Ganglia Thalamus | SB | II | Mut | Mut | TT | No | Yes: (8 months of follow up) |
| 13   | 34  | Male | Aphasia | Left Fronto-Temporo-Insular | SB | II | Mut | Wt | TC | No | Yes: (12 months of follow up) |
| 18   | 60  | Female | Aphasia | Multicentric: Left Frontal and two Right Frontal | SB | III | Wt | Mut | TT | No | No: 10 months after diagnosis: ICP (Intracranial pressure) |
| 1    | 61  | Female | Hemiparesis | Subcortical Right Rolandic | SB | IV | Wt | Wt | TT | Yes | No 3 months ICP (Intracranial pressure) |
| 3    | 66  | Female | Seizures | Left Temporal | SB | IV | Wt | Mut | CC | No | No 4 months (Septic shock urinary focus) |
| 4    | 75  | Male | Walking Instability | Butterfly wings Posterior Corpus Callosum | SB | IV | Wt | Wt | CC | Yes | No 2 months (ICP) |
| 6    | 78  | Female | Visual disorders | Subcortical Left Occipital | SB | IV | Wt | Wt | TT | No | Yes: 6 months of follow up |
| 7    | 42  | Male | Cephalea Visual disorders | Subcortical Right Temporal | XS | IV | Mut | Mut | CC | No | Yes: (18 months of follow up) |
| 9    | 67  | Female | Hemiparesis | Subcortical Right Fronto-Parietal | SB | IV | Wt | Wt | TC | Yes | No 6 months (ICP) |
| 11   | 55  | Male | Behavioral disorders Aphasia | Subcortical Left Frontal | XS | IV | Wt | Wt | TC | No | Yes: (15 months of follow up) |
| 14   | 53  | Female | Aphasia | Subcortical Left Frontal | XS | IV | Wt | Mut | CC | Yes | Yes: (8 months of follow up) |
| 16   | 43  | Female | Cephalea, Nausea, Vomiting (IPC) | Subcortical Right Frontal and Corpus Callosum | XS | IV | Wt | Mut | TT | Yes | No: 3 months (ICP) |
| 17   | 74  | Male | Visual disorders | Subcortical Right Temporo-Occipital | SB | IV | Wt | Wt | CC | Yes | No 4 months (ICP) |
| 19   | 32  | Female | Cephalea and Visual Disorders | Subcortical Left Occipital | XS | IV | Wt | ND | ND | Yes | Yes: 11 months of follow up |

WHO grade was determined by histopathological analysis. Grade II: Low-grade gliomas; Grade III and IV: High-grade gliomas; SB: Stereotaxic biopsy; XS: Surgery for total resection of tumor; Mut: mutated; Wt: wild type; ND: Not detected; SNP: single-nucleotide polymorphism; ICP: Intracranial pressure.
Frequency of IDH1 mutations compared with patients’ characteristics.

An example of the mutation at codon 132 of IDH1 shown in Figure 1B. The following characteristics studied:

i) Glioma grade: Most of the patients with low-grade glioma presented mutated IDH1 and, most of the patients with high grade-glioma presented wild type (wt) IDH1 (Table 2).

The mean age of the patients with mutant IDH1 was 35±7 years, whereas the mean age of the patients with wt IDH1 was 56±18 years (p=0.00372).

ii) Tumor growth: increased tumor size was observed in only one patient with mutated IDH1 (Odds Ratio: O.R. 0.083, IC 95%: 0.007-0.949), whereas most patients with the wt IDH1 presented tumor growth in the same period (O.R. 12.12, IC 95%: 1.05-136.8) (Table 2). Results of the odds ratio (O.R.) indicate that mutated IDH1 is a protective factor and that a wt IDH1 denotes 12.12 times more risk for tumor growth in 6 months. The Chi-Square assay with the Yates corrections resulted in a significant p-value (* p=0.0419). Therefore, the tumor growth is associated with wt IDH1.

Table 2: Histopathological classification of gliomas, tumor growth and IDH1 status

| IDH1 status | Histopathological classification of gliomas | Odd Ratio and CI 95% | Tumor growth detected By NMR in 6 months | Tumor growth IDH1 Mut vs Wt | P value |
|-------------|--------------------------------------------|----------------------|------------------------------------------|----------------------------|---------|
|             | Low-grade | High-grade                 |                        | Growth | No growth |                        |          |          |
| IDH1 Mut    |          |                           | 0.083, CI: 0.007-0.95 | 1 | 6           |                        |          |          |
| IDH1 Wt     |          | 1 | 11                        | 12.12, CI: 1.05-136.8 | 8 | 4           |                        | 0.0419   |

iii) Survival: six patients out of the 12 with wt IDH1 survived less than six months, whereas all the seven patients with a mutated IDH1 survived all the studied period (Figure 1C). Kaplan-Meier analysis and the Log-rank (Mantel-Cox) statistic showed a significant difference p=0.02 (data not shown). Considering patient’s age, the seven patients who did not survive during the studied period had an average age of 64 years, in contrast, the 12 patients who survived had an average age of 40 years, this difference being significant (p=0.02).

Figure 1: Patients’ clinical features and IDH1 mutations; A) Age distribution of patients grouped in 10-year intervals; B) IDH1 sequence showing the double pick of heterozygous mutation in the 132 codon. C) Time of survival of patients with wild type and mutant IDH1. Arrows indicate the patients with wild type IDH1 who died within the first six months (6 cases) and between seven and twelve months (1 case); D) Magnetic Resonance (MRI) image of the cerebral hemispheres (case #5) shows a bithalamic injury (left) and tumor growth after 6 months of treatment (right).
An example of histopathological analysis and IDH1 status compared with clinical characteristics in a 16-year-old patient: The histopathological test revealed grade II diffuse astrocytoma (no necrosis or extended vascularization), whereas the molecular data indicated a wt IDH1, which was associated with a severe clinical course in this patient and a large tumor including both thalami and the base ganglia. The patient evolved with tumor growth detected at six months by MRI, as shown in Figure 1D.

**TERT mutations and polymorphism**, Sixteen tumors studied for TERT promoter mutations, six mutations found at the sites -124bp, five heterozygous C>T transitions, and at -146bp, one homozygous C>T substitution, which denotes Loss of Heterozygosity (LOH). None of the constitutional leukocyte DNA samples showed these mutations. Regarding the rs2853669 polymorphism at -245bp of TERT promoter, eight patients (50%) were TT genotype, and eight were TC and CC (Table 1). The correlation of constitutional geno
type with tumor genotype showed LOH in two grade-II gliomas and one Grade-IV glioma, all with mutated IDH1 (data not shown). No significant association between TERT promoter mutation and polymorphism status found, although there was a trend towards the prevalence of T allele in the gliomas with wt TERT (Table 1).

**TERT mutations and clinical characteristics.** i) Age and glioma grade: No significant difference in age found between patients with or without mutation in the TERT promoter, 49±12 years and 53±22 years, respectively. The mutated TERT promoter found in 5 high-grade gliomas and 1 grade II glioma; in this tumor, the mutant T base was 50% of C base height, which denotes mosaicism (not all tumor cells harbored TERT mutation). On the other hand, the wt TERT was present in 6 high-grade and four low-grade gliomas (Figure 2A). Fisher’s exact test was not significant (p=0.08); there was no association between tumor grade and TERT mutation. However, there was a higher proportion of mutated TERT than wt TERT in high-grade gliomas.

ii) Tumor growth and survival of patients with a mutation in TERT pro
tomer: No difference in tumor growth observed between the presence and absence of C>T transition in the TERT promoter (p=0.60) (Table 1). However, a higher proportion of patients with TERT promoter mutation died (3 out of 6 patients) compared to the proportion of patients with wt TERT (4 out of 10 patients) (Figure 2B). Survival analysis showed a lower, although not significant, survival of patients with mutated TERT after ten months (p=0.82, data not shown).

**TERT promoter polymorphism and clinical characteristics.** i) Glioma grade: Twelve polymorphic C alleles were present in high-grade gliomas and 1 C allele in lower-grade gliomas (Figure 2C). Fisher exact test showed a significant value (p=0.008). Therefore, the presence of the C allele was associated with a high glioma grade. Also, a higher proportion of C allele than T allele was present in gliomas with mutated TERT compared to gliomas with wt TERT (3CC / 2TT vs. 2CC, 2TT, and 2TC, Table 1).

**Mutation status of TERT and IDH1:** Four out of 6 high-grade gliomas with mutated TERT exhibited wt IDH1. The other two gliomas displayed a mutation in both genes, one a grade II, with a mosaic TERT mutation, and Grade-IV glioma, which could be a secondary glioblastoma derived from IDH1 mutant lower grade. On the other hand, 3 out of 5 gliomas with mutated IDH1 presented wt TERT and grade II (Table 1).

**Discussion**

Classification of gliomas according to molecular markers is better than the histopathological diagnosis for understanding the biology and prognosis of gliomas (Louis et al., 2016). The mutational status of the IDH1 gene is one of the most used molecular markers; the somatic hotspot mutation in the highly conserved substrate-bind
domain frequently occurs in grade II gliomas. The presence or absence of IDH1 mutations has crucial prognostic value in anaplastic gliomas and glioblastomas since these mutations are associated with a more favorable clinical course (Parsons et al., 2008). The beneficial effects of mutated IDH1 are due to epigenetic reprogramming induced by the mutated IDH1 enzyme. Changes in the tumor immune microenvironment can lead to non-immune suppressive neutrophils, correlated with favorable patient outcomes (Castro et al., 2020). On the other hand, alteration in the redox system with an accumulation of free radicals can lead the tumor cell to oxidative stress causing DNA and cellular damage (Lee et al., 2002). Our results confirm these findings since only one patient with mutated IDH1 showed tumor growth, but this patient had a p53 mutation and a cellular proliferation index Ki67 of 15% (case #2). Conversely, the wild-type status of IDH1 is associated with a severe clinical course. Thus, a patient with a wt IDH1 presented an extended tumor size and grave symptoms despite a histopathological classification as grade II glioma (case #5). Of note, four patients with a wt IDH1 and no tumor growth have received radiotherapy 60 days after diagnosis, which may reflect the importance of early treatment with radiotherapy.
Patients’ age is relevant for progression of the disease, as have been shown by other reports (Parsons et al., 2008, Eckel-Passow et al., 2015), and our results. Patients older than 60 years had a worse survival than younger patients, and none of those showed mutated IDH1, the average age of patients with wt IDH1 was significantly higher than the average age of patients with mutated IDH1. Therefore, our results show that the evolution of the disease may be predicted primarily by the presence or absence of IDH1 mutation and secondarily by other factors: i) in cases with mutated IDH1, the other factors may be mutations in other markers (as was observed in patient #2); ii) in subjects with a wt IDH1 the evolution depends on patient’s age.

Mutations in the TERT promoter that generate binding sites for specific transcription factors originate a two to four-fold increase in TERT expression (Killela et al., 2013). TERT mutations have been reported in high-grade gliomas with a frequency of 48% to 84% (Killela et al., 2013; Park et al., 2014; Simon et al., 2015) and less frequently in lower-grade gliomas (Killela et al., 2013; Ramakrishna et al., 2015). The rs2853669 polymorphism in the TERT promoter may also have a prognostic value (Spiegs-Kreinecker et al., 2015, Batista et al., 2016). We found a low frequency of TERT mutations in high-grade gliomas; however, none of the low-grade gliomas showed mutated TERT (except one with a mosaic mutation). Regarding clinical parameters related to TERT mutational status, there was no significant association between tumor growth/survival and the mutated TERT. However, survival analysis showed a trend toward a lower survival of patients with mutated TERT, but the small number of samples preclude statistical significance for these variables. The presence of polymorphism in the TERT promoter can also influence the outcome of gliomas. Our results show an association of the polymorphic C allele with a high tumor grade and a trend towards a higher proportion of C allele in gliomas with mutated TERT and patients with lower survival.

Figure 2: TERT mutations and polymorphism in different clinical presentations: A) TERT mutations in low and high glioma grade; B) TERT mutations and patients’ survival; C) TERT polymorphism and glioma grade. C allele in low-grade vs high-grade gliomas * p<0.05; D) TERT polymorphism and patients’ survival at different times after diagnosis.
Regarding the relation between the mutation status of IDH1 and TERT, our results were consistent in that most of the TERT mutated high-grade gliomas showed wt IDH1 and that the majority of IDH1 mutated low-grade gliomas showed wt TERT, suggesting that IDH1 and TERT mutations could be mutually exclusive. Evaluation of the mutation status of the TERT and IDH1 genes in gliomas provides higher reliability to the prognosis of the disease (Yang et al., 2016; Vuong et al., 2019).

The success of treatment predicted by the status of molecular markers, also may serve as targets for future gene therapy. Concerning this issue, Li et al. (2020) demonstrated that the annihilation of mutations in TERT promoter by gene editing inhibits brain tumor growth. Though the clinical utility is yet uncertain, molecular studies are a crucial step to understand biological mechanisms which rule the evolution of gliomas.

Conclusion

This work shows that the use of genetic markers is essential for prognosis in patients with gliomas. IDH1 mutations in gliomas are frequent in Indian, Chinese, and other populations, as have been reported, indicating an association between IDH1 mutational status and the outcome in gliomas. Our results support the use of IDH1 mutational status as a marker for prognosis in Argentine patients. Association of the TERT mutational status with glioma-grade, though without statistical significance, is useful for future studies on glioma-markers. Our data are the first reported, in this context, in literature from Argentina.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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