CDKN2A promoter hypermethylation in astrocytomas is associated with age and sex

Markênia Kélia Santos Alves a, *, Mário Henrique Girão Faria a, Eduardo Henrique Cunha Neves Filho a, Adriana Camargo Ferrasi b, Maria Inês de Moura Campos Pardini b, Manoel Odorico de Moraes Filho c, Silvia Helena Barem Rabenhorst a

a Universidade Federal do Ceará, Department of Pathology and Forensic Medicine, Rua Alexandre Baraúna, 949, Porangabussu, CEP 60183-630 Fortaleza, Brazil
b Universidade Estadual Paulista - UNESP, Botucatu Medical School, Molecular Biology Laboratory of Blood Transfusion Center, Distrito de Rubião Junior, s/n, CEP 18.618-000 Botucatu, Brazil
c Universidade Federal do Ceará, Department of Physiology and Pharmacology, Rua Coronel Nunes de Melo, 1127, Porangabussu, CEP 60430-270 Fortaleza, Brazil

Article info
Article history:
Received 14 February 2013
Received in revised form 24 April 2013
Accepted 17 May 2013
Available online 27 May 2013

Keywords:
CDKN2A Methylation
p16
Expression
Age
Astrocytic tumors

ABSTRACT
CDKN2A promoter hypermethylation has been widely related to many cancers. In astrocytomas, although CDKN2A (p16INK4A protein) is often inactivated, there are still some controversial issues regarding the mechanism by which this alteration occurs. Thus, we analyzed a series of astrocytomas to assess the association between CDKN2A expression and methylation of grade I–IV tumors (WHO) and clinico-pathological parameters. DNA extracted from formalin-fixed paraffin-embedded material of 93 astrocytic tumors was available for CDKN2A promoter methylation analysis and p16 INK4A expression by methylation-specific PCR and immunohistochemistry, respectively. A strong negative correlation between nuclear and cytoplasmic immunostaining and CDKN2A promoter methylation was found. Additionally, a significant negative correlation between CDKN2A promoter methylation and age was observed; also, female patients had statistically more CDKN2A methylated promoters (p = 0.036) than men. In conclusion, CDKN2A inactivation by promoter methylation is a frequent event in astrocytomas and it is related to the age and sex of patients.

© 2013 Surgical Associates Ltd. Published by Elsevier Ltd. All rights reserved.

1. Introduction
The astrocytomas are the most common primary brain tumors, being classified according to their histological degree of malignancy by the World Health Organization (WHO) in grades I–IV. These categories result from the recognition of anaplasia findings, such as nuclear atypia, cell pleomorphism, mitotic activity, endothelial hyperplasia and necrosis.1,2

Individually or in combination with genetic mechanisms, epigenetic alterations, such as aberrant hypermethylation of promoter regions, affect the expression of tumor suppressor genes and DNA repair genes, leading to their silencing.3 Blocked expression of tumor suppressor genes or impairment of their products may result in loss of proliferative control, leading to the neoplastic process in the development of many human tumors.4

In astrocytomas, besides p53 and MGMT [a specific predictive biomarker of tumor responsiveness to chemotherapy with alkylating agents5], the p16INK4A (cyclin-dependent kinase inhibitor 2A-CDKN2A) protein, encoded by the CDKN2A gene, is often inactivated.6,7 This protein is a cell cycle regulator involved in the inhibition of the formation of cyclin D1/cyclin-dependent kinase 4 or 6 complexes during the G1 phase (Gap1) of the cell cycle.8 Although, in most human tumors, gene promoter hypermethylation is a common mechanism of CDKN2A inactivation, in astrocytomas there are still some controversial issues.9–13 Also, few studies have evaluated this process considering the expression and the methylation status of this gene taking in account clinicopathological aspects, such as histological grade and age.9,14 Additionally,
there are controversial issues concerning the nuclear cytoplasmic localization of the p16\textsuperscript{INK4A} protein expression.\textsuperscript{15–17}

Therefore, in this study, we performed an analysis of CDKN2A expression and methylation status in a series of astrocytic tumors of different grades (WHO) from the state of Ceará, Brazil. We also investigated the relationship between these aspects and clinicopathological parameters.

2. Material and methods

2.1. Ethical issue and casuistry

The present study was approved by the Ethics Committee of the Hospital Complex of the Federal University of Ceará under protocol 32/2004, respecting the Resolution 196/96 of the National Council of Health – Ministry of Health/Brazil. Ninety-three formalin-fixed paraffin-embedded astrocytic tumors from the archives of the BIOPSE\textsuperscript{TM} (Biomédica, Pesquisas e Serviços Ltda. – Fortaleza, CE – Brazil), a private pathology laboratory which is reference in this field in our region, selected from the routine histopathological examinations performed between 2003 and 2011, were examined. The samples were sliced at 5 μm thickness and processed for histopathological examination (hematoxylin–eosin stain), DNA extraction (for MS-PCR) and immunohistochemical analysis.

2.2. DNA extraction from paraffin-embedded specimens

All specimens included in this study were taken from paraffin-embedded tissues. Ten sections from each sample were obtained from the blocks with adequate precaution to prevent contamination between cases, including replacement of blades between each block. Microtome holders were cleaned using xylene between the blocks. Microtome holders were cleaned using xylene between

2.3. Sodium bisulfite treatment and methylation-specific PCR (MS-PCR)

DNA extracted from paraffin-embedded tumor tissue was modified by sodium bisulfite to determine the methylation status of the CDKN2A gene by MS-PCR, as previously described by Ferrarei et al.\textsuperscript{18} The primers targeted to the promoter CDKN2A region were 5′-TTATTAGAGGGTGGGCGGATCGC-3′ (sense) and 5′-GACCCCGAACCGGACCGTAA-3′ (antisense) for the methylated CDKN2A (151 bp), and 5′-TTATTAGAGGGTGGGCGGATCGC-3′ (sense) and 5′-CAACCCCAACCAACCAACCA-3′ (antisense) for the unmethylated CDKN2A (151 bp). PCR was performed in 25 μL reaction volume, containing 1× Platinum Taq buffer, 3.0 mM MgCl\textsubscript{2}, 0.4 mM of each dNTP, 0.64 mM of each primer set, 1 U Platinum Taq DNA Polymerase\textsuperscript{TM} (Invitrogen, Foster, CA, USA), and 50 ng of treated DNA.

DNA methylated in vitro by Sss-I methylase\textsuperscript{20} (New England Biolabs, Beverly, MA, USA) was used as a positive control. Water and DNA from peripheral lymphocytes of healthy donors were used as negative controls. The PCR products were resolved in a 6% non-denaturing polyacrylamide gel and subsequently submitted to silver staining.

2.4. Immunohistochemistry

Immunostaining was performed according to a previously described protocol.\textsuperscript{19} For antigen retrieval, deparaffinized sections were pretreated by heating in a microwave oven in 10 mM citrate buffer, pH 6.0, for 20 min. After cooling, sections were immersed in PBS containing 3% hydrogen peroxide for 10 min to block endogenous peroxidase activity. Sections were then incubated in a moist chamber overnight at 4 °C with p16 primary antibody (clone 6H12; dilution 1:40; Novocasta, United Kingdom). After rinsing with PBS, the slides were incubated with secondary antibody followed by streptavidin–biotin-peroxidase complex (LSAB + system; DakoCytomation\textsuperscript{®} USA), both for 30 min at room temperature with a PBS wash between each step. The reaction was revealed with diaminobenzidine–H\textsubscript{2}O\textsubscript{2} (DAB + system; DakoCytomation\textsuperscript{®}, USA), counterstained with Harry’s hematoxylin and mounted. A confirmed case of nuclear p16-positive human breast carcinoma was used as a positive control. Controls of primary antibody specificity included omission or substitution of primary antiserum by normal bovine serum.

2.5. Immunostaining analyses

The immunohistochemical evaluation for p16 was performed independently by two experienced technicians, using direct light microscopy. Any conflicting results were jointly considered for a consensual determination. Protein expression was quantified by manual counting of at least 1000 tumor cells in 10 different fields at a magnification of ×400. The labeling index (LI) expresses the percentage of nuclear or cytoplasmic positive cells in each tumor sample and H-score takes into account the intensity of the cytoplasmic p16 stain expressed in values ranging from 0 to 3 (0 – no stain, 1 – weak, 2 – moderate and 3 – strong), following the methods described by McCarty et al.\textsuperscript{20} Only cases with LI equal to or greater than 5% were considered positive.

2.6. Statistical analyses

The statistical analyses were carried out using the SPSS version 15.0 program (Chicago, IL, USA). Statistically significant differences were evaluated by the chi-square test (χ\textsuperscript{2}) or Fisher’s exact test. Correlations between immunostaining and CDKN2A promoter region methylation status were analyzed by Spearman’s rank correlation coefficient. The results were considered statistically significant when the ρ-values were less than 0.05.

3. Results

3.1. Patient characteristics

A total of 93 samples of formalin-fixed paraffin-embedded astrocytic tumors of different grades (WHO classification), 17 (18.3%) grade I, 19 (20.4%) grade II, 14 (15.1%) grade III, and 43 (46.2%) glioblastomas were included in this study. The median age of the patients was 42 years (range, 2–81 years). A significant positive correlation (p = 0.000; R = 0.677) was observed between age and grade of malignancy of tumors. Among the cases, 50 were men and 43 women.

Fig. 1. Immunohistochemical staining for p16\textsuperscript{INK4A} protein in formalin-fixed paraffin-embedded astrocytic tumors (×400). [A] Pilocítico Astrocytoma (WHO Grade I): moderate nuclear and cytoplasmic staining; [B] Glioblastoma (WHO Grade IV): diffuse staining.
3.2. Frequency and correlation between methylation in promoter regions and expression of CDKN2A

Nuclear and/or cytoplasmic p16\textsuperscript{INK4A} positivity was found only in tumor cells. No staining was seen in non-tumor specimens. Examples of immunostaining for p16\textsuperscript{INK4A} and promoter methylation CDKN2A are illustrated in Figs. 1A,B and 2. The presence of promoter methylation in CDKN2A was observed in 65.6% (61/93) of the cases and expression of nuclear and cytoplasmic p16\textsuperscript{INK4A} protein were observed in 26.5% (25/93) and 51.6% (48/93) of the cases, respectively. The concurrence (+/+) and agreement (+/+ or −/−) of nuclear and cytoplasmic expression of p16\textsuperscript{INK4A} occurred in 20.4% (19/93) and 62.4% (58/93) of the analyzed cases, respectively. A high significant negative correlation was found between p16\textsuperscript{INK4A} nuclear or cytoplasmic immunopositivity and CDKN2A promoter region methylation (p < 0.001; \( R = -0.378 \) and p < 0.001; \( R = -0.384 \), respectively).

3.3. Relationship among promoter methylation and expression of the CDKN2A, age and sex of the patients

Fig. 3[A]–[B] shows the frequencies of CDKN2A promoter methylation and p16\textsuperscript{INK4A} expression stratified according to age and sex of the studied patients. From these figures, it is possible to observe that besides a relevant frequency of CDKN2A promoter methylation found in all age intervals, the younger patients (<30 years old) had the highest frequencies of methylated CDKN2A when compared to patients aged ≥ 30, with statistical significant (p = 0.002). Indeed, correlation analysis showed a significant negative correlation between CDKN2A promoter methylation and age (p = 0.047, \( R = -0.206 \)). Additionally, female patients had statically more CDKN2A methylated promoters (33/43; 76.7%; p = 0.036) than men (Fig. 3[A]). No significant correlation between patient age and sex and p16\textsuperscript{INK4A} expression (nuclear or cytoplasmic) was found (Fig. 3[B]).

3.4. Relationship between promoter methylation and expression of CDKN2A and histological grade of tumors

The distribution of the p16\textsuperscript{INK4A} expression and methylation status according to tumor grade is showed in Table 1 and Fig. 4. The high frequency of 16\textsuperscript{INK4A} nuclear negative cases was specially observed among the diffuse grade tumors. Additionally, grade I astrocytic tumors demonstrated median values of nuclear LI significantly higher (Median LI: 3.0) than diffuse tumors (grades II to IV) (Median LI: 0.0) (p < 0.01) (Fig. 4).

Conversely, when analyzing the expression of cytoplasmic p16\textsuperscript{INK4A}, it was observed (Table 1 and Fig. 4) that the frequency of positivity, the median value of cytoplasmic LI and the H score was positively correlated to histological tumor grade (p = 0.001, \( R = 0.337 \); p < 0.001, \( R = 0.386 \); p < 0.001, \( R = 0.375 \), respectively). Also, grade IV tumors showed higher LI and HS median values than the other grades, with statistical significance (p < 0.001).

No correlation between tumor grade and CDKN2A promoter methylation was observed (Table 1).

4. Discussion

In many human tumors, the inactivation of the tumor suppressor p16\textsuperscript{INK4A} protein by promoter hypermethylation of its codifying gene is a frequent event, and it has been associated with the carcinogenesis process.\textsuperscript{21,22} Up to date, there are only few studies on astrocytomas addressing mechanisms that may lead to p16 inactivation, presenting controversial results.\textsuperscript{8,9,11,23–25} Additionally, these studies did not evaluate the data taking the immunopositivity and the methylation status of the CDKN2A gene combined according to histological and clinical aspects.

Similar to other studies,\textsuperscript{11,26} our study found that only 26.5% of the tumors showed nuclear p16\textsuperscript{INK4A} immunopositivity. Additionally, the positivity of p16\textsuperscript{INK4A} was higher in pylocytic tumors than in the diffuse ones, which could represent a way to hinder the tumorigenic process, since those tumors present benign features, with minor molecular modifications.\textsuperscript{1,27} On the other hand, 51.6% of the tumors were p16\textsuperscript{INK4A} cytoplasmic immunopositive. The concurrence or agreement maintained between p16\textsuperscript{INK4A} nuclear and cytoplasmic staining indicates that cytoplasmic staining was specific and not artifactual. According to Liang et al.,\textsuperscript{28} cytoplasmic staining indicates the accumulation of this protein in the cytoplasm, caused by its phosphorylation mediated by protein kinase B, oncogenetically activated, which blocks the recognition of its sites by nuclear importation factors. In this study, p16\textsuperscript{INK4A} cytoplasmatic staining was positively correlated to tumor grade, suggesting that the greater genomic instability of the higher grade tumors\textsuperscript{27} could lead to the expression of p16\textsuperscript{INK4A}; however, due to the acquisition of new molecular modifications, p16\textsuperscript{INK4A} nuclear importation could be blocked, explaining our findings.

Homozygous deletion has been described as the inactivating mechanism of the CDKN2A gene, in gliomas. However, some studies have found CDKN2A promoter methylation in astrocytic tumors.\textsuperscript{8,12,13,25,28} Among the studies related to the CDKN2A promoter methylation in astrocytomas, some publications found a very low
rate of methylation, or failed to find it.\textsuperscript{9,12,13} In our study, a high frequency of methylation in \textit{CDKN2A} promoter was observed in about 70\% of tumors of all grades, except for grade IV tumors, which dropped to 60\%, in agreement with other studies.\textsuperscript{8,11} Methylation in the \textit{CDKN2A} promoter appears to be the mechanism blocking \textit{p16INK4A} expression in astrocytomas, since a direct strong negative correlation between immunohistochemical expression and methylation was observed. These data confirm that methylation in the promoter regions appears to be the main mechanism of gene silencing, as indicated in studies by Park et al.\textsuperscript{11} and Alves et al.\textsuperscript{22} and that mutation does not appear to be a common phenomenon in astrocytomas.\textsuperscript{12}

To investigate the factors that may influence promoter methylation in astrocytomas, we next correlated clinical patient characteristics with \textit{CDKN2A} expression and \textit{CDKN2A} promoter methylation status. In this study, there was a striking correlation between age at diagnosis and gender and \textit{CDKN2A} promoter methylation, with a predominance of methylated \textit{CDKN2A} in young female patients. Few reported studies focusing on methylation in astrocytic tumors have taken into account sex and age. Yu et al.,\textsuperscript{9} in a study of methylation in astrocytomas, did not find this relation-ship for \textit{CDKN2A}, and besides, hypermethylation of the AR gene was found to occur frequently in females. Li et al.,\textsuperscript{30} studying genes other than \textit{CDKN2A}, found an association between N33 and ER methylation in older glioblastoma patients but not in younger patients, although both of these genes are more often methylated in normal elder individuals than in the younger ones. As gene promoter methylation pattern changes with age and hormonal status,\textsuperscript{31–35} it seems reasonable to take into account both parameters in the analysis. Alternatively, triggering factors (genetic predisposition, carcinogen exposure) may be significantly different between the young and older group of patients with astrocytomas, and these factors may then influence the incidence of \textit{CDKN2A} methylation.

In summary, \textit{CDKN2A} inactivation by promoter methylation is a frequent event in astrocytomas and is related to age and sex of patients. Our data suggest important interplay between gene methylation and aging and sex in tumorigenesis, but future studies should address the functional significance of the methylation events observed in astrocytomas.

\textbf{Ethical approval}

The present study was approved by the Ethics Committee of the Hospital Complex of the Federal University of Ceará under protocol 32/2004.

\textbf{Funding}

None.

\textbf{Author contribution}

Alves, MKS: Study design, data collections, data analysis and writing.

Faria, MHG: Study design, data collections, data analysis.

Neves Filho, EHC: Data collections.