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# IMMUNOEXPRESSION OF TUMOR SUPPRESSOR GENES *P53*, *P21$^{WAF1/CIP1}$* AND *P27$^{KIP1}$* IN HUMAN ASTROCYTIC TUMORS

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ABSTRACT - The aim of the present study was to evaluate the tumor suppressor genes *p53*, *p21$^{WAF1/CIP1}$* and *p27$^{KIP1}$* expression in astrocytic tumors, correlating the findings with the histopathological grade (WHO). An immunohistochemical study of the p53, p21 and p27 proteins using the streptavidin-biotin-peroxidase method was performed in fifty-five astrocytomas (13 grade I, 14 grade II, 7 grade III and 21 grade IV) and five samples of non-tumor brain tissue (negative control). p53 positive indices (PI) and labeling indices (LI) showed tendency to increase according to malignant progression. The nuclear expression of p27 presented similar inclination, except for the PI reduction verified in grade IV tumors. Otherwise, the cytoplasmic p27 staining was more evident between high-grade tumors (III and IV). p53 and nuclear p27 expression was correlated with the histological classification (p<0.01; test *H*). On the other hand, p21 indices revealed a propensity to reduction in agreement with malignant evolution of the astrocytic tumors, except for high scores observed in grade IV tumors. The non-tumor samples did not show any expression of these proteins. These results indicated the *p53* mutation as an initial, relevant and potentially predictor of tumor progression event in astrocytomas, with the detection of p21 protein as an important resource for the deduction of functional situation of this gene. Moreover, the activation of *p27$^{KIP1}$* was preserved in the astrocytic tumors and its cytoplasmic manifestation seems to be resultant of its nuclear expression, not demonstrating a direct impact in astrocytomas tumorigenesis.

KEY WORDS: astrocytoma, glioblastoma, tumor suppressor genes, immunohistochemistry.

**Imuno-expressão dos genes supressores tumorais *p53*, *p21$^{WAF1/CIP1}$* e *p27$^{KIP1}$* em tumores astrocíticos humanos**

RESUMO - O presente estudo objetivou avaliar a expressão dos supressores tumorais *p53*, *p21$^{WAF1/CIP1}$* e *p27$^{KIP1}$* em tumores astrocíticos humanos, correlacionando os achados com a graduação histopatológica (OMS). Procedeu-se o estudo imuno-histoquímico para as proteínas p53, p21 e p27 utilizando o método da estreptavidina-biotina-peroxidase em 55 astrocitomas (13 do grau I, 14 do grau II, 7 do grau III e 21 do grau IV) e 5 amostras de tecido cerebral não-tumoral (controle negativo). Os índices de positividade (PI) e de marcação (LI) para p53 demonstraram tendência de aumento conforme a progressão maligna. A expressão nuclear do p27 apresentou semelhante inclinação, exceto pela redução do PI verificada nos tumores do grau IV. Já a marcação citoplasmática do p27 foi mais evidente entre tumores de alto grau (III e IV). As expressões de p53 e p27 nuclear demonstraram correlação com a classificação histológica (p<0,01; teste *H*). Por outro lado, os índices para p21 manifestaram propensão à redução conforme a evolução maligna dos tumores astrocíticos, salvo significante aumento observado nos tumores do grau IV. Não houve expressão dessas proteínas nas amostras não-tumorais. Tais resultados indicaram a mutação do *p53* como um evento inicial, relevante e potencialmente indicador de progressão maligna nos astrocitomas, sendo a detecção da proteína p21 um importante recurso para a dedução da situação funcional desse gene. Além disso, a ativação do *p27$^{KIP1}$* mostrou-se preservada nos tumores astrocíticos e sua manifestação citoplasmática parece ser reflexo de sua expressão nuclear, não demonstrando impacto direto na tumorigênese dos astrocitomas.

PALAVRAS-CHAVE: astrocitoma, glioblastoma, genes supressores tumorais, imuno-histoquímica.

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Astrocytomas constitute the main histological type among the primary tumors of the central nervous system (CNS). Although corresponding only to 1.32% of all neoplasms annually diagnosed, astrocytic tumors represent the most frequent solid tumor in childhood and the second cause of death by cancer in this age group, behind only leukemia. In adults, the mean five-year survival rate for these tumors is approximately 32%, constituting the ninth and tenth cause of cancer mortality among men and women, respectively[1]. The World Health Organization (WHO) classifies astrocytomas according to different grades of malignancy (I to IV). Basically, these categories result from the recognition of anaplasia findings (nuclear atypia, cell pleomorphism, mitotic activity, endothelial hyperplasia and necrosis) through routine histological analysis by light microscopy[2]. As an overall rule, the accumulation of anaplastic findings seems to reflect the progression of molecular disorders acquired during the neoplastic transformation[3]. Among them, alterations of cyclin-dependent kinase (CDK) inhibitor genes, jointly known as tumor suppressors in association with *p53* and *Rb* genes, emerge as an important pathway. The class of tumor suppressor proteins is considered to be the main responsible for the strict regulation of the cell cycle. In this way, the blockade of the expression or the impairment of the performance of these molecules may result on the lack of control of the proliferative function, leading to the neoplastic process[4]. The tumor suppressor gene *p53* is located on chromosome 17p13.1. Its inactivation, usually caused by mutation of a single copy followed by allelic loss of the remaining chromosome, was one of the first identified events in the astrocytomas tumorigenesis, being described in approximately 60% of all astrocytic tumors[5]. The p53 protein acts basically as a transcription factor, activating regulatory molecules of many cellular programs, including cell cycle, response to the DNA damage, apoptosis, cell differentiation and angiogenesis. In the genetic damage response, for example, the p53 protein can induce the expression of GADD-45, which promotes (1) the cell cycle arrest in the G2 phase, probably through the association with the cyclin B/CDK1 complex, (2) the blockade of the replication, through the formation of suppressing complexes with the PCNA and (3) the feebleness of the DNA-histones interactions, enabling the DNA repair[6]. The wild-type p53 protein is slightly expressed in normal cells, having a short half-life (20-30min). However, its mutant version (inactive) is highly stable, promoting its cell accumulation and thus making its detection possible. For this reason, the immunohistochemical method has been proposed as a simple and quick way of investigating *p53* mutations[5,7].

The main transcriptional target of the p53 is represented by the *WAF1* gene (also known as *CIP1*, *SDI1*, *mda-6* or *CDKN1A*), located on chromosome 6p21.1. This gene encodes a phosphated protein of 21kDa, which also exhibits tumor suppressing activity, denominated $p21^{WAF1/CIP1}$. The p21 protein acts as a negative regulator of the cell cycle, inhibiting the CDKs activity on G1-S transition and controlling the DNA synthesis (S phase) through the blockade of PCNA[8]. The direct regulation of the *WAF1* expression by p53 implicates that, in the presence of the mutation of the last one, the levels of p21 would be dramatically reduced or totally absent. Thus, the p21 expression would reflect the functional status of the *p53* gene in a simple and accessible manner in association to detection of the mutated p53 protein.

Another tumor suppressor protein from the CIP/KIP family corresponds to $p27^{KIP1}$, encoded by the gene located on chromosome 12p13. The p27 protein presents 42% of structural homology with the p21, which justifies the similarity of these proteins on the blockade of the cell cycle progression through the inhibition of the cyclin D/CDK4, cyclin E/CDK2 and cyclin A/CDK2 complexes[9].

An intriguing fact consists on the detection of the p27 protein, a remarkable nuclear regulator, in the cytoplasm of neoplastic cells. Recent evidences suggest that oncogenetic expression of kinases such as PKB would promote the p27 protein phosphorylation, resulting in its cytoplasmic redistribution[10]. The presence of p27 in the cytoplasm would trigger its degradation process through ubiquitination followed by proteomic complexes digestion. Thus, the sequestration and destruction of p27 protein would warrant the continuous formation of cyclin/CDK complexes, promoting the cell cycle and, therefore, collaborating with the tumorigenic process[11].

In this context, the present investigation aimed to evaluate the expression of tumor suppressor genes *p53*, $p21^{WAF1/CIP1}$ e $p27^{KIP1}$ in human astrocytic tumors of different histopathological grades (WHO).

## METHOD

*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. We examined fifty-five formalin-fixed paraffin-embedded astrocytic tumors of different grades (WHO) [13 grade I, 14 grade II, 7 grade III and

21 grade IV] from the archives of the BIOPSE® (*Biomédica, Pesquisas e Serviços Ltda.* – Fortaleza, CE - Brazil), referring to the selected routine histopathological examinations performed during the period between 1999 and 2004. As a parameter of normalcy, five formalin-fixed paraffin-embedded samples of non-tumor brain cortex obtained from the Department of Pathology and Forensic Medicine (Medicine School - Federal University of Ceará) were used. The samples were sliced at 5 μm thickness and processed for histopathological evaluation (hematoxylin-eosin stain) and immunostaining.

*Immunostaining* – Immunostaining was performed according to a previously described protocol[12]. For antigen retrieval, deparaffinized sections were pretreated by heating in a microwave oven in 10 mM citrate buffer, pH6.0, 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 humid chamber overnight at 4°C with the following primary antibodies: p53 (clone DO-7; dilution 1:80; DakoCytomation®, USA), WAF1 (clone 4D10; dilution 1:40; Vision-Biosystems®, UK) and $p27^{KIP1}$ (clone SX53G8; dilution 1:100; DakoCytomation®, USA). After rinsing with PBS, slides were incubated with secondary antibody followed by streptavidin-biotin-peroxidase complex, both for 30 min at room temperature with a PBS wash between each step (LSAB+ system; DakoCytomation®, USA). The slides were developed with diaminobenzidine-$H_2O_2$ (DAB+ system; DakoCytomation®, USA), counterstained with Harry's hematoxylin and mounted.

*Histopathological and immunostaining analyses* – The immunohistochemical evaluation, as well as histopathological reviews, was performed by three experienced analysts independently using direct light microscopy. The eventual conflicting results were jointly considered for a consensual definition. The proteins expression was quantified through manual counting of at least 1,000 astrocytic cells in 10 different fields at a magnification of x400. The positive index (PI) represented the percentage of tumors positive for the antigens studied in each group (histological grade). The labeling index (LI) was expressed as the percentage of positive cells for nuclear or cytoplasmic staining in each sample[13]: The H-score (H) takes into account the intensity of the cytoplasmic p27 stain expressed in values ranging from 0 to 3+ [0 (no stain); 1+ (weak); 2+ (moderate) and 3+ (strong)], following methods described by McCarty et al.[14].

*Statistical analysis* – Descriptive statistics (means, standard deviations, and frequency distributions) was determined for continuous and categorical variables. Non-parametric approaches (Shapiro-Wilk test, Mann-Whitney U test, Kruskal-Wallis H test) were performed wherever applicable using SPSS® 14.0 software. The results were mainly expressed as means ±S.D. (standard deviation). $p<0.05$ was considered to be statistically significant.

## RESULTS

Examples of immunostaining for p53, p21 and p27 proteins are illustrated in Figure 1. The expression of these proteins was not evidenced in non-tumor astrocytes. The general positivity for p53, p21 e p27 (nuclear) in studied astrocytic tumors was 54.98%, 34.61% and 80.72%, respectively. The p53 positive indices (PI: 46.15% for grade I; 50.00% for grade II; 57.14% for grade III; 66.66% for grade IV) and mean scores (LI: 7.69; 9.64; 15.28; 22.95) demonstrated a tenuous propensity to increase in agreement with histopathological grade of the astrocytic tumors. The positive indices for nuclear p27 (PI: 53.84%; 92.86%; 100.00%; 76.19%) presented a significant rise according to the

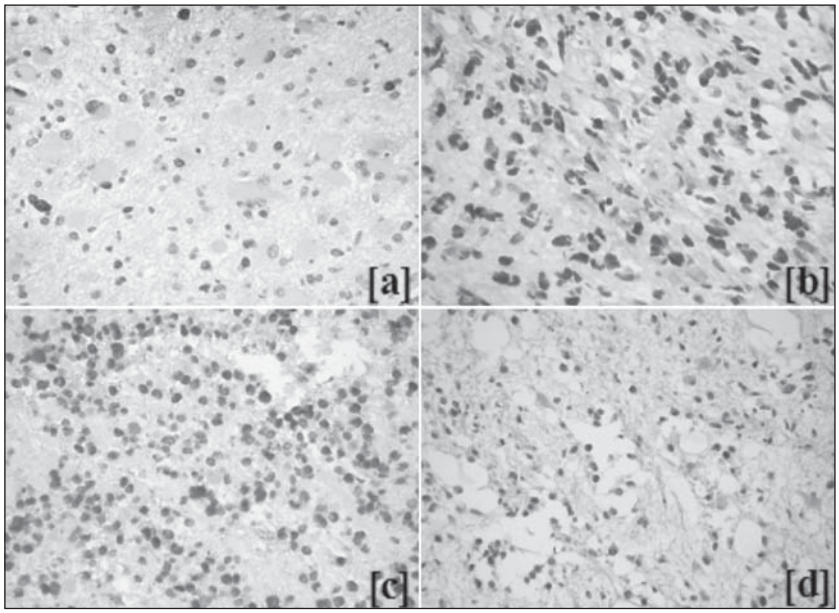


*Fig 1. Immunohistochemical staining for p53 [a, b], p21 [c] and p27 [d] proteins in formalin-fixed paraffin-embedded astrocytic tumors (x400). [a] Gemistocytic Astrocytoma (WHO Grade II): slight staining (LI=14); [b] Anaplastic Astrocytoma (WHO Grade III): diffuse staining (LI=60); [c] Glioblastoma (WHO Grade IV): diffuse staining (LI=80); [d] Fibrillary Astrocytoma (WHO Grade II): moderate nuclear (LI=40) and cytoplasmic (LI=20; H=30) staining.*

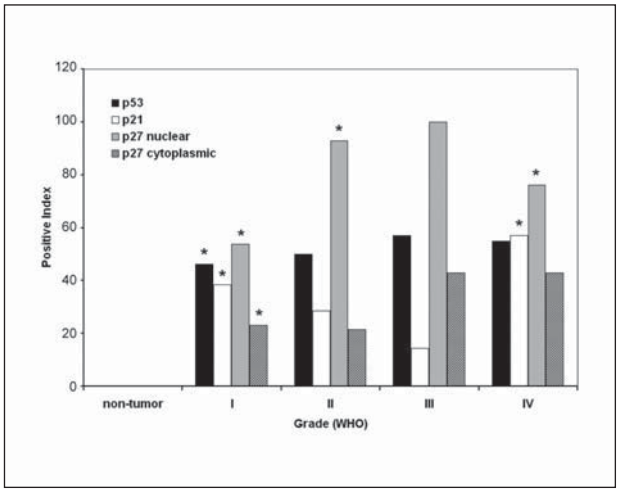


*Fig 2. Positive index of tumor suppressor proteins p53, p21 and p27 (nuclear and cytoplasmic) immunostaining according to histological classification of the cases studied (n=60). (*) p<0.05 when compared to previous group (Mann-Whitney U-test).*

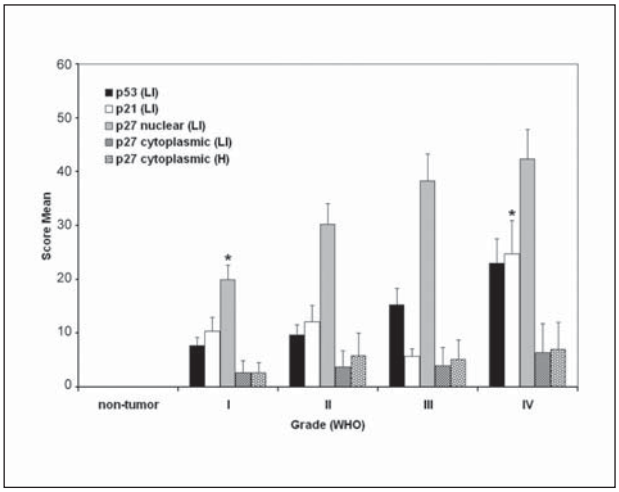


*Fig 3. Mean scores attributed to tumor suppressor proteins p53 (LI), p21 (LI), nuclear p27 (LI) and cytoplasmic p27 (LI and H) immunostaining according to histological classification of the cases studied (n=60). LI (Labelling Index); H (H-Score); (*) p<0.05 when compared to previous group (Mann-Whitney U-test).*

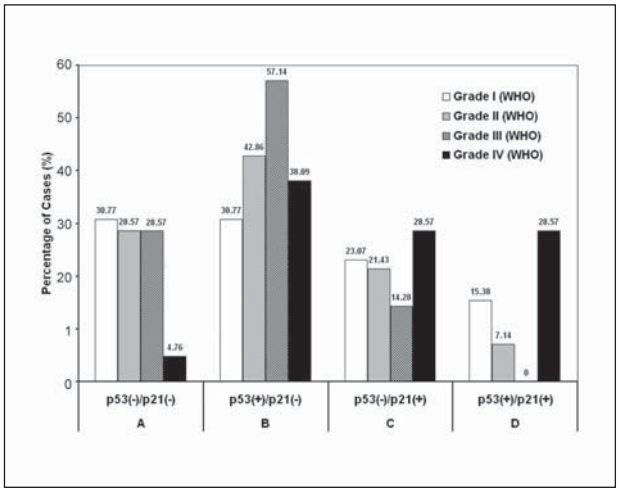


*Fig 4. Percentage distribution of different grades of astrocytic tumors according to the relationship between tumor suppressor proteins p53 and p21 expression (n=55). (+) immunopositive; (–) immunonegative.*

malignant progression, even though presenting a statistically significant reduction in the grade IV astrocytomas. However, the scores referring to its expression (LI: 20.00; 30.21; 38.28; 42.38) maintained the tendency of increasing according to the tumor grade, with a remarkable staining in glioblastomas (grade IV): about 40% of the cases presented positivity in more than 70% of the tumor cells (LI>70) (Figure 2 and 3). The staining for p53 and p27 (nuclear) was correlated in a significant manner with the histopathological classification (p<0.01; Kruskal-Wallis *H* test).

The cytoplasmic p27 staining was observed in 32.96% of the evaluated astrocytomas. It was verified a tender disposition of the cytoplasmic p27 expression to increase according to the tumor progression, being present more frequently in high-grade (III and IV: 42.86%) than in low-grade tumors (I and II: 23.07%). The mean score values for cytoplasmic p27 staining in

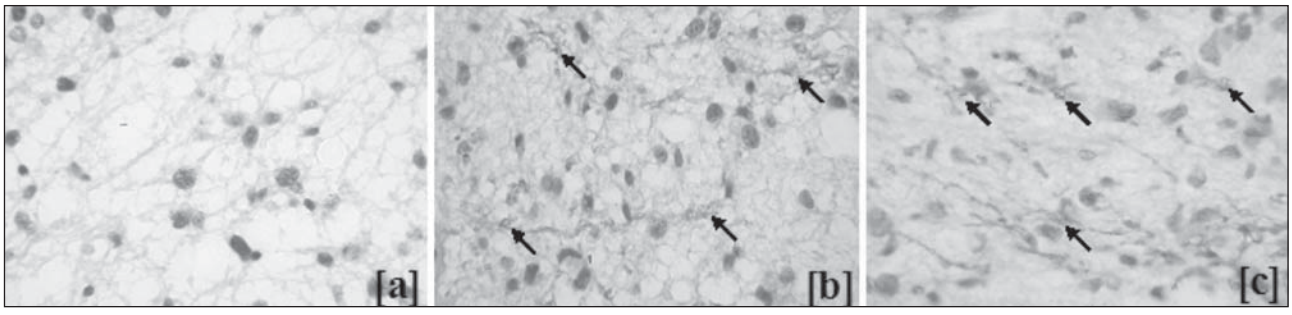


*Fig 5. Immunohistochemical staining for tumor suppressor proteins p53 [a], p21 [b] and p27 [c] detected in the same Pilocytic Astrocytoma (WHO Grade I) (x400): the expression of p53 in tumor astrocytes is followed by the positive staining for p21 and p27 in surrounding microglial cells (arrows).*

astrocytic tumors were slight, although having a propensity of increasing according to the tumor grade (H 2.69; 5.85; 5.14; 7.00 e LI: 2.69; 3.71; 4.00; 6.42) (Figure 3). The concomitancy (+/+) between the nuclear and cytoplasmic p27 detection demonstrated similar inclination: 42.86% in high-grade and 22.22% in low-grade tumors.

On the other hand, p21 indices manifested a tendency of reduction according to the histopathological grade up to grade III, contrasting with the significant increases in positive and expression scores observed for grade IV tumors (PI: 38.46%; 28.57%; 14.28%; 57.14% e LI: 10.38; 12.14; 5.71; 24.79). The relationship between the immunohistochemical expression of the tumor suppressors *p53* e *p21* (transcriptional target of *p53*) is presented in Figure 4, where it is noticed a small fraction (16.36%) of functional "disagreement" [represented by p53(+)/p21(+)], especially for Glioblastomas (grade IV).

Curiously, it was still observed the expression of p21 and p27 proteins in microglia surrounding the tumor cells in almost 70% of the positive cases for p53, notably in the high-grade astrocytomas (Fig 5).

## DISCUSSION

The present investigation demonstrated a discrete tendency of augmentation in the number of p53 positive cases according to the histopathological grade of the astrocytomas, showing resembling values to the reported by previous studies, assembled on Table 1. The mean labeling indices (LI) also presented the propensity to increase according to the astrocytic tumors progression (Fig 3), despite the similarity of the mean scores published by Nayak et al.[21] (0.00; 9.89; 8.15; 8.13).

The high values and the analogy between the labeling scores for p53 observed in different grades of astrocytic tumors reaffirms the *p53* gene mutation as an initial and relevant event in the formation of these neoplasms[5]. It is believed that the clonal proliferation of the *p53* mutated cells in conformity with tumor progression would extend the percentage of immunopositive cells[6], indicating the increase of the labeling indices (LI) here presented as more reasonable than the constancy described by Nayak et al. [21].

Among the exposed investigations (Table 1), we pointed out the conflicting results concerning the p53 protein expression in pilocytic astrocytomas (grade I). The data presented by the current investigation corroborate with the findings of Lang et al.[16], which describe the immunohistochemical detection of p53 grade I tumors, although in a higher percentage of the cases. This same tendency is ratified by a great number of studies that applied molecular methodologies (more sensible and specific) for detecting the *p53* gene alterations, confirming the occurrence of mutations of this gene in the tumorigenesis of pilocytic astrocytomas (Table 2). We considered that the success of *in situ* demonstration of p53 protein in grade I astrocytomas reported by Lang et al.[16] and by the present study has been motivated by the high technical performance achieved through the maximum optimization of the applied immunohistochemical method. However, future studies should definitively explain the impact of *p53* gene disorders in grade I astrocytic tumors, employing great tumor series examined by specific molecular approaches.

*Table 1. Positive index (PI) values for immunohistochemical p53 expression for different grades of astrocytic tumors according to various references, including the present study.*

| | Grade I (WHO) | | Grade II (WHO) | | Grade III (WHO) | | Grade IV (WHO) | | Total | |
|---|---|---|---|---|---|---|---|---|---|---|
| References | n | PI | n | PI | n | PI | n | PI | n | PI |
| Jaros et al.[15]*# | 4 | 0 | 6 | 17.00 | 13 | 38.00 | 20 | 65.00 | 43 | 44.00 |
| Louis et al.[5] | – | – | 8 | 37.50 | 12 | 58.33 | 14 | 50.00 | 24 | 50.00 |
| Lang et al.[16] | 7 | 71.00 | 8 | 63.00 | 16 | 64.51 | – | – | 31 | 64.51 |
| Kordek et al.[17] | 8 | 0 | 9 | 44.40 | 9 | 33.30 | 30 | 53.30 | 56 | 41.00 |
| Ono et al.[8]* | – | – | 15 | 40.00 | 20 | 40.00 | 13 | 38.00 | 48 | 39.58 |
| Khalid et al.[18] | – | – | – | – | – | – | 57 | 52.63 | 57 | 52.63 |
| Kirla et al.[19] | – | – | – | – | 25 | 44.00 | 52 | 46.00 | 77 | 45.45 |
| Pardo et al.[20]# | – | – | – | – | – | – | – | – | 74 | 48.00 |
| Nayak et al.[21] | 15 | 0 | 38 | 52.63 | 29 | 48.27 | 70 | 50 | 152 | 45.39 |
| Faria et al.* | 13 | 46.15 | 14 | 50.00 | 7 | 57.14 | 21 | 66.66 | 55 | 54.98 |

n (number of cases); (*) statistical association with the histological grade; (#) statistical association with the survival.

*Table 2. Percentage values of p53 gene mutation for different grades of astrocytic tumors according to various references, including the present study.*

| | | Grades I and II (WHO) | | Grade III (WHO) | | Grade IV (WHO) | |
|---|---|---|---|---|---|---|---|
| References | Method | n | % | n | % | n | % |
| Mashiyama et al.[22] | SSCP | 6 | 0 | 3 | 66.66 | 10 | 10.00 |
| Fults et al.[23] | SSCP | 6 | 0 | 14 | 37.71 | 25 | 28.00 |
| Louis et al.[5] | SSCP+Seq | 8 | 37.50 | 12 | 33.33 | 14 | 28.57 |
| Wu et al.[24] | SSCP | 9 | 0 | 6 | 16.60 | 38 | 12.15 |
| Chozick et al.[25] | SSCP | 15 | 0 | 7 | 14.28 | 19 | 31.57 |
| Lang et al.[16] | SSCP+Seq | 7 and 8 | 14.00 and 25.00 | 16 | 18.75 | – | – |
| Rasheed et al.[26] | SSCP | 36 | 8.30 | 11 | 54.54 | 51 | 17.3 |
| Patt et al.[27] | SSCP | 7 and 18 | 14.30 and 5.06 | 4 | 25.00 | 13 | 0 |
| Ono et al.[8] | p53/p21 | 15 | 26.66 | 20 | 25.00 | 13 | 30.76 |
| | SSCP+Seq | | 26.66 | | 25.00 | | 23.07 |
| Hayes et al.[28] | SSCP | 20 | 53.00 | – | – | – | – |
| Kato et al.[29] | Seq | – | – | 14 | 64.30 | 27 | 25.90 |
| Faria et al. | p53/p21 | 13 and 14 | 30.77 and 42.86 | 7 | 57.14 | 21 | 38.09 |

n, number of cases; %, percentage of p53 mutation; SSCP, single–strand conformation polymorphism; Seq, sequencing; p53/p21, estimation according to the relationship between p53 and p21 expression.

Many researches also report the detection of the p53 protein in astrocytic tumors as a prognostic factor. Jaros et al.[15] and Pardo et al.[20] refer to p53 protein as an indicator of the malignant progression, low disease-free time after surgery and low general survival. On the other hand, Birner et al.[30] associated the p53 protein detection to a greater sensibility of grade IV tumors to radiotherapy and adjuvant chemotherapy.

The confirmation of the *p53* gene mutation as an important molecular disorder in astrocytomas also encouraging the research for tools able of restoring the gene functions in these tumors. Using the *p53* tumor suppressor gene restitution techniques through viral vectors (genetic therapy), Tsuchiya[31] observed greater radiosensitivity among the treated grade IV astrocytomas lineages, while Geoerger et al.[32] demonstrated the induction of apoptosis and reduction of the tumor growth in primary cultures and xenotransplants deriving from glioblastomas after transfection with wild-type *p53* gene.

Few references on the literature explored the role of the tumor suppressor gene *WAF1* (encoder of $p21^{WAF1/CIP1}$ protein) in the neoplastic process of the astrocytomas, since its mutation was described as a rare event among these tumors[8]. Nevertheless, the fact of *WAF1* being the main transcriptional target of the *p53* gene gives a new perspective for a better functional understanding of this inhibiting cell cycle pathway from the guided gene-expression (protein products) studies[10].

The analyses of the p21 protein expression in studied astrocytic tumors revealed a propensity of reduction in the percentage of the positive cases and in the mean labeling scores (LI) according to tumor malignancy, despite the notable increases of these indices in grade IV astrocytomas (Figs 2 and 3). Khalid et al.[18] and Kirla et al.[19], studying the *p21* expression in glioblastomas, describe positive indices of 57% and 48%, respectively. Ono et al.[8] verified similar inclination in the mean scores (LI) according to the tumor grade, in spite of the extension of this reduction to the grade IV tumor (22.2 for grade II, 14.0 for grade III; 10.2 for grade IV).

A preliminary evaluation of these findings lead to lower p21 expression according to the malignant progression of the astrocytic tumors, despite the intriguing increase of its expression in the grade IV astrocytomas. However, it is convenient to examine such results associated to the findings concerning to the p53 protein immunodetection (Fig 4).

Considering the incapability of the muted p53 protein to activate the p21 protein expression as well as the detection of this last one as an evidence of *p53* functionality, it was observed that, among the different tumor grades, similar percentages do not demonstrate alteration in the p53/p21 tumor suppressor pathway {groups A [p53(-)/p21(-)] and C [p53(-)/p21(+)]}, except for grade III tumors in group C and the grade IV tumors notably in group A. On the other hand, crescent percentages of astrocytomas present alterations in p53/p21 pathway according to the tumor pro-

gression {group B [p53(+)/p21(-)]}, in spite of the lower indices verified in the grade IV tumors. Different fractions of the astrocytomas exhibited a functionally conflicting activation of the p53/p21 pathway {group D [p53(+)/p21(+)]}, especially the grade IV tumors.

Group B represents the cases where an alteration of *p53* gene surely occurred. Increasing labeling scores confirms the previous findings of the incidence of *p53* mutation according to the tumor grade, suggested by the augmentation of p53 protein expression. The smaller fraction of the grade III tumor in group C reinforces the great impact of *p53* mutation in this tumor category. Also in group B, it was noticed a smaller percentage of grade IV tumors, despite the tendency to increase concerning the remarkable expression of p53 in this grade (Fig 3). Actually, we suggest that this conflict may be explained by the expression of the wild-type p53 protein in a portion of glioblastomas (grade IV). This expression would occur through the activation of this tumor suppressor caused by other genetic alterations present in grade IV tumors, leading to the accumulation of a notable quantity of functional p53 protein, which is able to persist in detectable amount in tissues[20]. This fraction would probably be represented by grade IV tumors found in group D, where the evidence of wild-type p53 detection is reinforced by the positivity for p21. Ono et al.[8] report that astrocytomas which accumulate the p53 protein in the absence of the *p53* gene mutations tended to present enhanced p21 expression compared to the ones which accumulate mutated p53 or did not store p53. Therefore, we suggest that the expressive mean increase in p21 protein expression verified in glioblastomas is, at least partially, reflex of the wild-type p53 superexpression in a portion of these tumors.

Another possibility for the percentage reduction referring to grade IV astrocytomas in group B concomitant to the augment in group D would be the superexpression of the MDM2 protein (inactivator of wild-type p53), resulting in little or none p21 expression despite the *p53* activation. However, previous studies make this alternative more remote since, besides the MDM2 gene amplification would be verified in only 10% of the studied glioblastomas, neither of these positive cases would have been detected among tumors with immunoexpression of the p53 protein associated to *p53* gene mutation[5].

It is admitted that the cases described in group D, including grades I and II astrocytomas, could still indicate the expression of p21 through independent pathways of the induced ones by *p53*. This possibility is based on the observation of p21 expression in knockout cells for *p53 gene*, triggered by cytotoxic agents, mitogens, inductors of cell differentiation, among others[33]. In astrocytomas, this phenomenon was evidenced in cell lineages *in vitro*[34] as well as in biopsy materials[8]. We suggest that other genetic alterations implicated in glioblastomas (grade IV) tumorigenesis act on mechanisms which are distinct from p53/p21 pathway, contextualizing the tumors categorized in group A.

The inference of the *p53* gene functional status from the immunohistochemical p53 and p21 proteins expression demonstrates a clear correlation with the detection of *p53* mutations by specific molecular techniques, although not dispensing them[8]. The use of this strategy by the present study allowed the deduction of *p53* gene mutation indices that follow the tendency of most part of the references (increase according to astrocytomas malignancy, except for the decrease observed in grade IV tumors), in spite of the great variability among the values pointed by different investigations (Table 2).

With great structural and functional homology for p21, emerges yet the tumor suppressor $p27^{KIP1}$. Quantitative alterations of p27 protein are frequently described in human tumors, despite the *p27* gene mutations and deletions are rarely observed[9]. Recent evidences indicate that these alterations would be regulated by exclusively post-transcriptional mechanisms, as protein degradation mediated by ubiquitines, and would influence directly on the tumor biological behavior[11].

Few studies refer to the *p27* expression in astrocytomas. Piva et al.[35] and Mizumatsu et al.[36] report the inverse correlation between the astrocytic tumors grading and the mean nuclear labeling indices (LI) for p27 [(44.40 for grade II; 5.86 for grade III; 2.10 for grade IV) and (64.0; 54.9; 48.0; 40.2), respectively]. On the other hand, Alleyne et al.[37] and Zagzag et al.[38] did not evidence any relationship between the astrocytomas malignancy graduation and the p27 immunoexpression. However, the present investigation disclosed crescent positive indices for p27 (nuclear) in astrocytomas according to the tumor grade, except for the relative reduction in grade IV tumors (Fig 2). The mean scores for the nuclear p27 staining demonstrated a discrete propensity to augmentation in accordance with the astrocytic tumor grade (Fig 3). The highest mean score verified in grade IV astrocytomas, in spite of the low positive index, reflects the expres-

sive labeling indices (LI>70) verified in about 40% of glioblastomas.

Notwithstanding the remarkable divergences among different studies, the present results indicate that the p27 nuclear expression in astrocytomas occurs proportionally to the tumor grade, reflecting the functional activation of *p27* as an inhibiting mechanism of the neoplastic process. However, among the grade IV astrocytomas, significant percentages of cases did not express p27, demonstrating once more the heterogeneity of this group. We suggest that these tumors present activated pathways of p27 protein degradation, since higher cytoplasmic p27 levels were not evidenced (see ahead) and the *p27* gene mutation is recognizably uncommon among human tumors. In this way, we perceive that alterations on the nuclear p27 protein expression only reflect the tumor malignancy, not demonstrating any impact in astrocytomas tumorigenesis.

Most researches which evaluate the p27 expression in human neoplasms disregard the detection of this protein in the cytoplasm, referring to this as destitute of functional meaning[38]. However, new studies reveal that recent translated p27 protein can be intercepted immediately before the reentrance in the nucleus through phosphorylation mediated by protein-kinase B (PKB or Akt), oncogenetically activated. Consequently, the cytoplasm accumulation of p27 would represent the functional blockade of its functioning as tumor suppressor (nuclear), stimulating the neoplastic progression[11].

The present study configures the first analyses of the cytoplasmic p27 protein expression in astrocytic tumors. The results indicate higher positive indices for cytoplasmic p27 among the high-grade astrocytomas related to low-grade tumors (Fig 2). At the same time, the concomitance between the nuclear and cytoplasmic p27 staining was more evident in high-grade tumors (42.86%). The parallel increase of the nuclear and cytoplasmic p27 staining in conformity with the progression of astrocytic tumors, associated to a higher concomitance of positive indices in high-grade tumors, suggests that the cytoplasmic p27 expression in astrocytomas reflects the nuclear expression of this protein, at least partially.

However, the mean values for cytoplasmic p27 staining scores in the astrocytic tumors were modest, even with a slight propensity of increasing according to the tumor grade (Fig 3). These low cytoplasmic p27 labeling scores may indicate that (1) the cytoplasmic p27 protein blockade occurs in small amounts or (2) an efficient p27 protein degradation is verified after its sequestration in the cytoplasm. In any way, the cytoplasmic presentation of p27 protein does not seem to be relevant for the tumorigenic process of astrocytomas.

Although current evidences did not indicate the participation of *p27* in the promotion of astrocytic tumors, Mizumatsu et al.[36] e Kirla et al.[39] describe the low expression of p27 as an indicator of unfavorable prognosis. Chen et al.[40] reported the transfection of the functional *p27* gene in gliomas as a promising method for cell proliferation inhibition, while Park et al.[41] related better results using the mutated *p27* gene. Otherwise, some analysis points the *p27* as an inducer of tumor resistence[42]. Lloyd et al.[9] indicated yet the potential involvement of *p27* in many mechanisms, as the cell differentiation, apoptosis, cell interaction/adhesion and inflammation. In such case, new researches will be necessary for the establishment of the exact role of this tumor suppressor protein in the astrocytomas tumorigenesis.

Furthermore, we noticed a frequent staining for p21 and p27 among the microglial cells surrounding to the astrocytic tumor tissues immunopositives for p53 (Fig 5), especially in high-grade tumors. The tumor suppressor proteins expression in microglia was already described after trauma injury and even in the peritumoral regions of the astrocytomas[43,45]. However, for the first time, it is established the relationship among this staining and the presence of a specific molecular change in the tumor tissue. In spite of the intracellular autonomy related to the direct control of the proliferative mechanisms, we suggest that in some way the microglia may react to the presence of molecular disorders in the tumor cells[44]. Future studies should elucidate this phenomenon, as well as evaluate its possible impact in the biological behavior of astrocytic tumors.

In conclusion, the presented results confirm the *p53* mutation as an initial and relevant event, as well as a potential indicator of malignant progression in the astrocytomas. The detection of the p21 protein represented an important resource for deduction of the functional situation of the *p53* gene, while the functional activation of *p27* does not seem to be damaged by the neoplastic process in the astrocytic tumors. Besides, the remarkable presence of the p21 protein among the glioblastomas (grade IV) revealed the superexpression of the wild-type p53 protein and/or the participation of other molecular disorders distinct from the p53/p21 pathway in a fraction of these

tumors. The cytoplasmic p27 expression was characterized as a reflex of its nuclear expression, not demonstrating any impact in the astrocytomas tumorigenesis. Finally, we emphasize that the identification of the main molecular alterations which are involved in the astrocytomas tumorigenesis gives new perspective not only to the diagnosis outline and clinical prediction but, above all, to the elaboration of target therapy, representing a new era in the oncology practice, where the individual and molecularly guided approaches will define the better antineoplastic therapy.

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