

# Relationship between the Presence of the R337H Mutation of the *TP53* Gene and Expression of the p53 and p53R2 Proteins in Choroid Plexus Carcinomas

Short title: R337H mutation and p53/p53R2 expression in choroid plexus carcinomas

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**Abstract:** *Background:* The most common mechanism associated with the development of choroid plexus carcinoma seems to be mutation of the *TP53* gene. The R337H mutation of the *TP53* gene has been reported in a significant proportion of patients with choroid plexus tumors. The aim of the present study was to use immunohistochemical techniques to establish whether the presence of the R337H mutation is associated with changes in the expression of p53 and p53R2 in choroid plexus tumors. *Methodology/Principal Findings:* A total of 27 samples collected from patients with choroid plexus tumors were screened for p53 and p53R2 expression using immunohistochemistry; the results were compared as a function of the presence or absence of the R337H mutation. Immunopositivity for p53 was more frequent in patients with choroid plexus carcinomas than in patients with choroid plexus papillomas ( $p=0.006$ ). All six cases of choroid plexus papilloma (100%) tested positive for the p53R2 protein. Patients with choroid plexus carcinomas that were immunopositive for p53R2 had higher odds of survival ( $p=0.041$ ). The R337H mutation and tissue immunopositivity of p53 could be used in the differential diagnosis of atypical papillomas because both occurrences are strongly associated with choroid plexus carcinomas. The use of these factors would make it unnecessary for the World Health Organization (WHO) to maintain a category for atypical papilloma in their classification system. Some evidence points to an association between absence of the p53R2 protein and progression to death in cases of choroid plexus carcinoma. *Significance:* Our results show an additional biomarkers that can aid in the diagnosis, treatment and determination of the prognosis of patients with choroid plexus carcinoma will continue to drive future research.

**Keywords:** choroid plexus carcinoma; p53; R337H; p53R2

## 1. Introduction

Choroid plexus tumors represent 0.4 to 0.6% of all intracranial neoplasms and predominantly affect children less than three years old [1-3].

According to histological criteria, choroid plexus tumors are classified as choroid plexus papillomas (WHO grade I) or choroid plexus carcinomas (WHO grade III) or as intermediate forms known as atypical choroid plexus papillomas (WHO grade II). Application of molecular biology techniques might allow for differential diagnoses among these three grades and consequently improve the treatments and prognoses of patients [4].

Although the ratio of the occurrence of choroid plexus carcinoma to papilloma is 1:5, Bleggi-Torres *et al* (2004) found an increased incidence of choroid plexus carcinoma in Southern Brazil [5,6].

The etiology of choroid plexus tumors is unknown; however, it is believed that the mechanism most commonly associated with the development of choroid plexus carcinomas is dysfunction of the *TP53* tumor suppressor gene, which encodes the p53 protein [7-9]. A particular mutation of the *TP53* gene was detected in a significant proportion of Brazilian patients with choroid plexus tumors. This mutation occurs in exon 10 and is known as R337H; it leads to a base-pair substitution of G to A, with a corresponding change in codon 337 from CGC (arginine) to CAC (histidine). The arginine residue of codon 337 is part of an important alpha-helix involved in the functional oligomerization of the p53 protein. By replacing arginine with histidine, the R337H mutation hinders the final stage of p53 oligomerization, disrupting its function. This mutation was first identified in Brazilian children with adrenocortical carcinomas [10,11]. Custódio *et al* (2011) detected the R337H mutation of *TP53* in 63% of choroid plexus carcinomas analyzed in Southern Brazil [12]. In addition, the survival of patients without the R337H mutation and those patients who survive surgery is longer, even when radiotherapy is not performed [4].

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Another molecule relevant to carcinogenesis and directly related to p53 is the protein known as p53R2. This protein is homologous to one of the components of the ribonucleotide reductase (RR) system, which provides nucleotides for DNA repair during the cell cycle. The presence of p53R2 in tumors is significant because its expression is regulated by p53 in response to DNA damage.

The aim of the present study was to establish whether the presence of the R337H mutation of *TP53* is associated with changes in the expression of immunohistochemical markers for p53 and p53R2 in choroid plexus tumors.

## 2. Materials and Methods

### Study population

The present study included 27 patients with choroid plexus tumors subjected to craniotomy at the Little Prince Children's Hospital (Hospital Infantil Pequeno Principe) from 1992 to 2010. Six cases were diagnosed as choroid plexus papillomas, and 21 cases were diagnosed as choroid plexus carcinomas. The description of this population and the methods used for DNA extraction and *TP53* genotyping are described in Custódio *et al* [12].

The present study is part of a larger research project titled "Neonatal screening, mapping of the prevalence of the R337H mutation in *TP53* according to county, cancer history, socioeconomic profile and molecular abnormalities associated with familial tumors in the state of Paraná" that was approved by the Research Ethics Committee (Comitê de Ética em Pesquisa - CEP) of the Little Prince Children's Hospital and the National Council of Research Ethics (Conselho Nacional de Ética em Pesquisa/CONEP - 677/2009).

### Immunohistochemical reactions

Representative areas of the tumors were selected, and two cylinders, 1 mm in diameter each, were taken from all of the tumor samples to prepare tissue microarrays (TMAs). This step was performed using the Manual Tissue Arrayer (MTA-I) (Beecher Instruments Inc., Sun Prairie, Wisconsin, USA).

The immunohistochemical reactions were automated using the BenchMark ULTRA (Ventana Medical Systems, Inc., Rotkreuz, Switzerland). The slides were identified using barcode labels and processed by the BenchMark ULTRA. Deparaffinization was performed with EZ PREP solution (Ventana Medical Systems, Inc., Rotkreuz, Switzerland), and heat-induced antigen retrieval was carried out using the Cell Conditioning 1 (CC1) solution (ULTRA CC1 Solution, Ventana Medical Systems, Inc., Rotkreuz, Switzerland) at high pH and 96 °C for 15 minutes.

Blocking of endogenous peroxidase was then performed for five minutes using a peroxidase blocking reagent (ultraView Universal DAB Inhibitor - 3% H<sub>2</sub>O<sub>2</sub>, Ventana Medical Systems, Inc., Rotkreuz, Switzerland) followed by a rinse with wash buffer. The sections were then incubated with anti-p53 (Clone DO-7, Dako, Glostrup, Denmark) and anti-p53R2 (N-16, Santa Cruz Biotechnology, Dallas, Texas, USA) antibodies (1:300) for 30 minutes.

The sections were incubated with horseradish peroxidase (HRP) polymer (HRP Multimer, Ventana Medical Systems, Inc., Rotkreuz, Switzerland) and rinsed several times with wash buffer. The sections were then incubated with the chromogen diaminobenzidine (DAB), rinsed with wash buffer and counterstained with hematoxylin (Hematoxylin II, Ventana Medical Systems, Inc., Rotkreuz, Switzerland). The slides were dehydrated in ethanol and xylene and mounted using a Tissue-Tek® Film® Coverslipper (Sakura, Alphen aan den Rijn, The Netherlands).

Reading of the anti-p53 and anti-p53R2 immunostained slides was performed on 10 large magnification fields (400x) with an Olympus BX50 Microscope (Tokyo, Japan) using the modified Allred score. Cells with nuclear staining for p53 and cytoplasmic/nuclear staining for p53R2 were considered to be positive. Thus, the samples were scored using the following system: 0 – negative, 1 – weak immunopositivity, 2 – moderate immunopositivity or 3 – strong immunopositivity. The values corresponded to the averages of 10 large magnification fields.

### Statistical analysis

For the purpose of statistical analysis, samples with staining intensities of 0 and 1 were considered to be immunonegative for p53 and p53R2, and samples with intensities of 2 and 3 were considered to be immunopositive for p53 and p53R2. In addition, the outcomes "alive" or "dead" were analyzed for choroid plexus carcinoma cases. The Chi-square and Fisher's Exact tests, which compare two sets of data, were used for statistical analysis. The significance level was set at  $p=0.05$ . The data were analyzed using SPSS v. 20.0 software (IBM Corporation, Armonk, New York, USA).

## 3. Results

### Investigation of the R337H mutation

The R337H mutation was detected in 61.7% of the patients with choroid plexus carcinomas ( $n=21$ ;  $p=0.016$ ). None of the cases of choroid plexus papilloma exhibited this mutation (Table 1).

**Table 1:** Absence or presence of the R337H mutation of *TP53* in the choroid plexus papilloma and carcinoma groups ( $n=27$ ).

R337H mutation	Choroid plexus papilloma* ( $n=6$ )	Choroid plexus carcinoma* ( $n=21$ )	<i>p</i> -value**
Absent	6 (100.0)	8 (38.1)	0.016
Present	0 (0.0)	13 (61.9)	

\*n (%); \*\*Fisher's Exact test

The outcomes "alive" or "dead" were analyzed as a function of the presence or absence of the R337H mutation in cases of choroid plexus carcinoma ( $p=1.000$ ). Approximately 54% of the patients with the mutation died, whereas no deaths occurred among the patients diagnosed with choroid plexus papillomas.

### Analysis of immunohistochemical results for p53

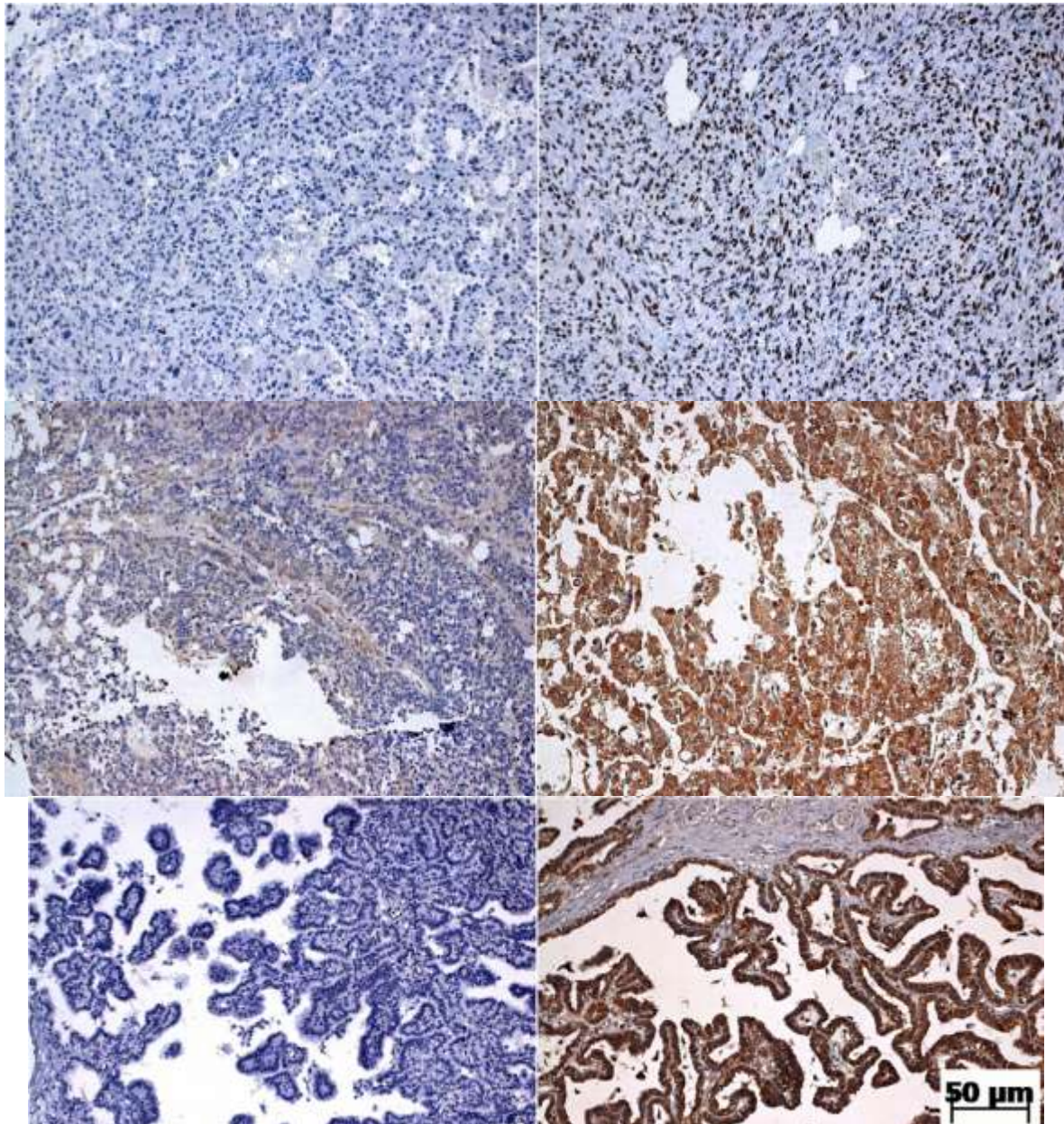
Table 2 describes the association between immunohistochemical detection of p53 expression and choroid plexus papillomas and carcinomas ( $p=0.006$ ). Figure

1 illustrates two cases of choroid plexus carcinoma (top); one case was positive for p53 (right), and one case was negative (left) for p53.

**Table 2:** Absence or presence of p53 and p53R2 expression (intensity) in the choroid plexus papilloma and carcinoma groups (n=27)

p53 intensity	Choroid plexus papilloma* (n=6)	Choroid plexus carcinoma* (n=21)	p-value**
Absent <sup>‡</sup>	6 (100.0)	7 (33.3)	0.006
Present <sup>¥</sup>	0 (0.0)	14 (66.7)	
<b>p53R2 intensity</b>			
Absent <sup>‡</sup>	0 (0.0)	5 (23.8)	0.555
Present <sup>¥</sup>	6 (100.0)	16 (76.2)	

\*n (%); <sup>‡</sup>no or weak staining; <sup>¥</sup>moderate or strong staining; \*\*Fisher's Exact test



**Figure 1:** Two cases of choroid plexus carcinoma (top); the one on the right is positive for p53, and the one on the left is negative for p53. Two more cases of choroid plexus carcinoma (middle) immunostained for p53R2; the one on the right is positive, and the one on the left is negative.

Finally, two cases of choroid plexus papilloma (bottom); the one on the right is positive for p53R2, and the one on the left is negative for p53. Notice the nuclear immunopositivity of p53 (top right) and the cytoplasmic/nuclear

immunopositivity of p53R2 (middle and bottom right). 400x magnification.

When considering only the patients diagnosed with choroid plexus carcinomas, no statistically significant relationship

( $p=0.346$ ) was found between the presence of the R337H mutation and a positive result for p53 expression (Table 3).

**Table 3:** Relationship between the presence of the R337H mutation of *TP53* and the expression of p53 and p53R2 proteins in choroid plexus carcinoma patients (n=21)

p53 intensity	Without R337H in <i>TP53</i> * (n=8)	With R337H in <i>TP53</i> * (n=13)	p-value**
Absent <sup>‡</sup>	4 (50.0)	3 (23.1)	0.346
Present <sup>¥</sup>	4 (50.0)	10 (76.9)	
p53R2 intensity			
Absent <sup>‡</sup>	2 (25.0)	3 (23.1)	1.000
Present <sup>¥</sup>	6 (75.0)	10 (76.9)	

\*n (%); <sup>‡</sup>no or weak staining; <sup>¥</sup>moderate or strong staining; \*\*Fisher's Exact test

No significant relationship was found ( $p=0.159$ ) between the outcomes "alive" or "dead" and the level of p53 expression in the group of patients with choroid plexus carcinomas (Table 4).

**Table 4:** Relationship between the outcome of death and the expression of p53 and p53R2 in choroid plexus carcinoma patients (n=21)

Outcome	p53R2 absent <sup>‡</sup> * (n=5)	p53R2 present <sup>¥</sup> * (n=16)	p-value**
Alive	0 (0.0)	9 (56.3)	0.041
Dead	5 (100.0)	7 (43.7)	
Outcome	p53 absent <sup>‡</sup> (n=7)	p53 present <sup>¥</sup> (n=21)	p-value**
Alive	5 (60.0)	4 (10.0)	0.159
Dead	2 (40.0)	10 (71.4)	

\*n (%); <sup>‡</sup>no or weak staining; <sup>¥</sup>moderate or strong staining; \*\*Fisher's Exact test

#### Analysis of immunohistochemical results for p53R2

All six cases of choroid plexus papilloma (100%) were positive for p53R2 (Table 2). Of the 21 cases of choroid plexus carcinoma, 16 cases (76.2%) were positive for p53R2 expression. Although interesting, this relationship was not statistically significant ( $p=0.555$ ). Figure 1 illustrates two cases of choroid plexus carcinoma (middle); one case is negative for p53R2 (left), and one case is positive for p53R2 (right). Figure 1 also depicts two cases of choroid plexus papilloma (bottom); one case is negative for p53 (left), and one case is positive for p53R2 (right).

Table 3 shows that there was no statistically significant relationship ( $p=1.000$ ) between the presence or absence of the R337H mutation and positive immunostaining for p53R2 in the group of patients with choroid plexus carcinomas (n=21).

Table 4 shows that there was a significant correlation ( $p=0.041$ ) between the expression of p53R2 and the outcomes "alive" or "dead" in the group of patients with choroid plexus carcinomas.

## 4. Discussion

This observational study investigated the expression of two important proteins, p53 and p53R2, and their relationship

with the presence of the R337H mutation in the *TP53* gene in patients with choroid plexus tumors.

In addition, our results provide preliminary evidence for the possibility of using molecular diagnoses that target the R337H mutation as a tool for the anatomical-pathological diagnosis of patients with choroid plexus tumors. Presence of the R337H mutation and immunopositivity for p53 might allow atypical papillomas and carcinomas to be distinguished in clinically ambiguous cases and thus help define cases with carcinoma. Such a definition is highly relevant for early clinical decision-making about the type of specific treatment required. There are different therapeutic approaches to papillomas and carcinomas. In cases of papilloma, surgical resection is the core treatment, and as a rule, end bloc resection is recommended as being curative [4,13]. However, patients with choroid plexus carcinomas should also receive adjuvant therapy after surgery. Some studies have shown that radiotherapy increases the survival of patients with choroid plexus carcinomas [3]. Nevertheless, radiotherapy should be avoided in pediatric patients because it could result in cognitive deficits, endocrine disorders and the development of secondary tumors. In that population, chemotherapy has been used as adjuvant therapy, and in some cases, it achieved a reduction in tumor size. An incorrect therapeutic approach could increase patients' morbidity and reduce their odds for longer survival.

Bleggi-Torres and colleagues found that the incidence of choroid plexus carcinoma was 63.2% of all choroid plexus tumors [5]. Custódio and colleagues reported that adrenocortical tumors and choroid plexus carcinomas are the most frequent neoplasms among patients under 15 years old [14]. In the present study, 77.7% of the choroid plexus tumors were carcinomas. This finding demonstrates that the incidence of choroid plexus carcinomas is high in the state of Paraná.

Tabori *et al* (2010) detected the somatic mutation of *TP53* in 50% of the cases of choroid plexus carcinoma but only in 5% of the cases of papilloma [9]. In 2013, Custódio *et al* reported that choroid plexus carcinomas exhibit the R337H mutation of *TP53* [14]. In our study (Table 1), nearly 62% of the cases with choroid plexus carcinoma carried this mutation, whereas none of patients with choroid plexus papilloma carried the mutation ( $p=0.016$ ). Based on this evidence, we suggest that choroid plexus carcinomas might be directly associated with the presence of the R337H mutation.

In 2012, Crocetti and colleagues reported that the five-year survival rate for choroid plexus carcinoma cases was 42.6%. This study significantly contributed to the understanding of patient survival; however, it did not assess the association between the survival rate and *TP53* mutations [15]. Some studies employed the R337H mutation as a prognostic factor and found that the survival time of patients with this mutation is short [4,9,13]. Our sample included four cases of choroid plexus carcinoma with the R337H mutation who are still alive more than five years after their initial diagnoses. This finding indicates that the R337H mutation of *TP53* might not be the only component of the signaling pathway

that increases the likelihood of the poorest outcome. Other types of molecules might interact with this mutation and play a role in increasing the survival of patients.

Tissue immunoexpression of p53 (Table 2) was positive in 66.7% of the choroid plexus carcinoma cases but in none of the papilloma cases ( $p=0.006$ ). More complete data are available in the study by Custódio *et al* [12]. Gugelmin (2005) found that 80% of choroid plexus carcinoma cases and 12% of papilloma cases were positive for p53 expression [17]. Expression of p53 was also observed by Tena-Suck *et al* (2010) in 86% of choroid plexus carcinomas and in 39% of papillomas [3]. Therefore, there is agreement in the literature that the expression of p53 is increased in choroid plexus carcinomas.

No significant association was found ( $p=0.346$ ) between the R337H mutation and tissue immunoexpression of p53 in the group of patients with choroid plexus carcinomas. A positive result for p53 expression was detected in 76.9% of the choroid plexus carcinoma cases carrying the R337H mutation, whereas 50% of the cases without the mutation were negative for p53.

No statistically significant association was found ( $p=0.159$ ) between the outcomes of patients with choroid plexus carcinomas and being immunopositive for p53. However, it is noteworthy that a large number of patients who progressed into the outcome “death” (71.4%) exhibited positive p53 expression in their tissues. Mutation of p53 inhibits its classic function as a tumor suppressor. Mutated p53 could negatively affect the regulation of cell differentiation, proliferation and apoptosis. Most *TP53* mutations cause changes between residues 100 and 300 in the DNA-binding domain [18]; as a result, mutated p53 may acquire new functions (“gain-of-function” phenomenon) with regard to cell migration, invasion and metastasis [19]. When p53 expression increases among choroid plexus carcinoma patients with the R337H mutation, it can be speculated that the half-life of this structurally altered p53 is increased, and thus, it is found more readily in cancer cells. In our study, the reason for the higher frequency of p53 expression among the cases that exhibited the outcome “death” might be related to a lack of early tumorigenesis suppression [20].

RR is a tetramer composed of two homodimers known as RRM1 (R1) and RRM2 (R2) [21]. The p53R2 protein is homologous to subunit R2 and contains a site for p53 binding. This binding stimulates the transcription/translation of a peptide that is notably similar to R2 [22]. Thus, p53R2 bound to R1 might play a role in DNA repair, mainly in the G1 and G2 cycle cell stages [16,23,24].

In the present study, all of the papillomas and 76.2% of the carcinomas were immunopositive for p53R2 ( $p=0.555$ ). We may infer that the presence of p53R2 was protective in patients with papillomas. This inference is based on the hypothesis that p53R2 provides the conditions for DNA repair by supplying deoxyribonucleotides (dNTPs). As a consequence, the papillomas do not become malignant. The ability of p53R2 to suppress tumor invasion in colon, oropharyngeal, pancreatic and prostate cancers is well

known in the literature. Okumura *et al* (2006) analyzed the relationship between expression of p53R2 in esophageal squamous cell carcinomas and tumor invasion, lymph node metastasis, pathological stage and p53 expression [25]. The group that was negative for p53 and positive for p53R2 exhibited deep tumor invasion and reached a more severe stage. Other authors found that high levels of p53R2 expression in melanoma is correlated with deep tumor invasion, distant metastases and tumor stage [26]. Such divergent functions of p53R2 could be related to mutations that affect this important protein. A point mutation that was detected in codon 115 of the *p53R2* gene in a cancer cell line (HCT116) leads to substitution of valine for leucine, with the consequent loss of p53R2 function [27]. In addition, Hsu *et al* (2011) assessed the expression of p53R2 in surgical specimens from lung cancer patients as a function of clinical parameters and survival rate [28]. The authors concluded that p53R2 expression was associated with higher survival rates; thus, it is associated with favorable prognoses and is a good biomarker for lung cancer.

Among the 13 patients in the present study that had choroid plexus carcinomas with R337H mutations, 10 (76%) exhibited positive expression of p53R2 (Table 3), although this association lacked statistical significance ( $p=1.000$ ). Table 4 shows that 100% of the choroid plexus carcinoma cases that were negative for p53R2 died and that 56% of the choroid plexus carcinoma cases that were positive for p53R2 survived ( $p=0.041$ ). The p53R2 protein supplies nucleotides for DNA repair; perhaps the occurrences of death among the individuals who were negative for p53R2 expression could be accounted for by loss of a DNA repair function [29]. Zhang *et al* (2011) suggested that p53R2 could suppress the proliferation of cancer cells independent of the status of p53 and thus represents a key element in anticancer therapeutics, especially in cases with *TP53* mutations [16].

## 5. Conclusion

The R337H mutation and tissue immunoexpression of p53 could be used in the differential diagnosis of atypical papilloma because both occurrences are strongly associated with choroid plexus carcinomas. Such a use would allow better and earlier therapeutic decision-making and would make the permanent inclusion of such an imprecise diagnostic category in the WHO classification unnecessary.

Although immunohistochemical assessment of choroid plexus papillomas and carcinomas showed that the former exhibited high levels of p53R2 expression and that the latter were positive for p53 and weakly positive for p53R2, new markers that have some joint activity with p53R2, such as p73, should be investigated because the roles of p53 and p53R2 cannot yet be asserted with full certainty. In addition, studies of p53R2 mutations would be very useful in the effort to elucidate the pathway in which the protein participates.

Available evidence points to a relationship between absence of the p53R2 protein and progression to death in cases of choroid plexus carcinoma. Further studies are needed to confirm this hypothesis, which is highly relevant for the

determination of new biomarkers with prognostic applications for these types of tumors.

Thus, the search for additional biomarkers that can aid in the diagnosis, treatment and determination of the prognosis of patients with choroid plexus carcinoma will continue to drive future research.

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