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ARTICLE

Simultaneous Alterations of Retinoblastoma and p53 Protein Expression in Astrocytic Tumors

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The genetic alterations frequently involved in glial malignancies are in the tumor suppressor genes, Rb and p53. An altered Rb expression or p53 overexpression is thought to indicate defective tumor suppression and subsequently more aggressive tumors. Therefore, to assess the alterations in the conjoint expression of Rb and p53 proteins in formalin fixed paraffin embedded sections, 64 astrocytic tumors were studied (16 astrocytomas, 7 gemistocytic astrocytomas, 19 anaplastic astrocytomas and 22 glioblastomas) using the avidin biotin immunoperoxidase technique. Fifty two cases (81.25%) were found to be positive for p53 protein. Seventeen of these showed aberrant heterogenous staining for pRb, of which 7 were glioblastomas. Only one case of astrocytoma showed aberrant expression of both p53 and Rb. Thus, of the 64 tumors, simultaneous aberrant expression of both p53 and Rb was seen in 21.9% of cases. This was more commonly observed among glioblastoma cases (7/22). No statistical difference was found between the survival rate of heterogenous pRb and p53 positivity in different grades of tumors. In glioblastomas, the survival rate appeared to be less in patients expressing heterogenous pRb, but this was not statistically significant. These results lead us to suspect that p53 and pRb pathways are inactivated, either through mutation or as part of the neoplastic process in astrocytic tumors. (Pathology Oncology Research Vol 5, No 1, 21–27, 1999)

Key words: astrocytic tumors, p53, pRb, immunochemistry, survival

Introduction

Alteration in oncogenes and tumor suppressor genes are now well documented to be associated with the genesis and progression of many human malignant tumors including gliomas.[5,24] Restriction fragment length polymorphism (RFLP) analysis of gliomas has revealed a non-random loss of heterozygosity (LOH) for markers on 5 chromosomes at the following loci 9p10, 10, 22q, 13p and 17p.[14] The latter two allelic losses have raised the question of involvement of the retinoblastoma (Rb) and p53 genes in the formation and progression of these tumors. The retinoblastoma (Rb) gene, the prototypic tumor suppressor gene, located at 13q14, encodes for a nuclear protein of 110 Kd[22] which is expressed by all normal tissues,[15] but is frequently seen in altered form in a variety of tumors. While germline mutations of this gene have been implicated in the development of hereditary retinoblastoma,[4] somatic mutations have been associated with a variety of human malignancies, most notably osteosarcomas,[12] soft tissue sarcomas,[20] bladder carcinomas,[19] and breast carcinomas.[29] In gliomas, specific evidence for Rb gene involvement was shown by Tsuzuki et al[30] who noted aberrations of the Rb gene in 3 of 23 astrocytomas (13.2%), all of which were Grade 4. It was also demonstrated that Rb gene inactivation and loss of its protein (p110 Rb) expression may be associated with glial tumor progression.[11,14]

Mutation of the p53 tumor suppressor gene located on chromosome 17p is the most frequent genetic abnormality identified in a wide range of human malignancies.[6] Loss of portions of the short arm of chromosome 17 has been found in gliomas, most of them being glioblastomas.[9,25] The p53 tumor suppressor protein is a nuclear phosphoprotein and mutations of the p53 gene often result in the accumulation of an abnormal mutant p53 protein. This mutated p53 protein has been demonstrated by immuno-

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histochemical staining in gliomas and strong correlation with malignancy grade has been established.

Though mutations of both Rb and p53 tumor suppressor genes and aberrant expressions of their proteins have been individually documented in gliomas, little is known about the relationship between these two genes in various grades of astrocytic tumors. The only reports are by Lee et al[23] who reported LOH on chromosome 10, Rb, 17p and p53 gene and Tsuzuki et al[30] who documented aberrations in the Rb, p53 and p16 gene in astrocytomas using PCR-SSCP analysis. To our knowledge, there is no report on the correlation of aberrant p53 protein expression and Rb protein expression in gliomas.

Hence the present study was undertaken to establish the incidence and pattern of Rb and p53 protein expression in a series of 64 astrocytic tumors of varying grades and to correlate the results with the histological grade and clinical outcome. The Rb and p53 protein expression were studied simultaneously in each tumor in order to determine whether there were specific types of alterations in their conjoint expression, and to assess whether such types of alterations were associated with specific tumor grades.

Materials and Methods

The tumor samples of 64 astrocytic tumors of varying grades were obtained at surgery from the Department of Neurosurgery, AIIMS, New Delhi with clinical information including age, sex, location of tumor, extent of resection, primary or recurrent tumor, treatment, overall survival and recurrence free survival. Survival time was computed from the date of diagnosis.

Histologic grading

Five micron sections stained for haematoxylin and eosin (H&E) were available in all cases. Histopathologic evaluation and tumor were reconfirmed by CS according to the new WHO classification.[21] The analysis revealed 16 astrocytomas, 7 gemistocytic astrocytomas, 19 anaplastic astrocytomas and 22 glioblastoma multiforme. Out of these 64, 4 were pediatric and the rest 60 were adult tumors.

Immunohistochemistry and assessment of Rb and p53 protein expression

A modified Avidin-Biotin Conjugate immunoperoxidase method of Bourne[2] was used for immunohistochemical staining. Five micron sections on gelatin coated slides were deparaffinised and rehydrated.

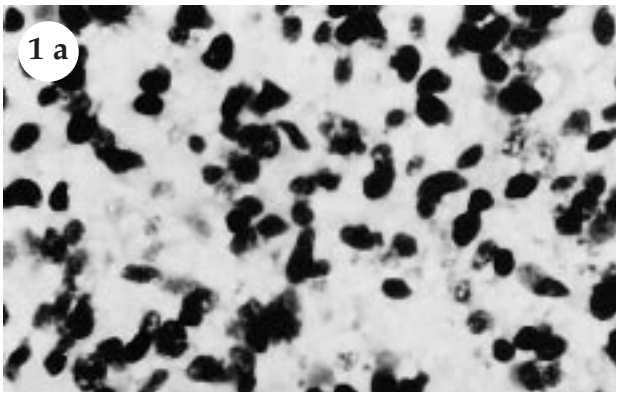
pRb – Endogenous peroxidase was blocked with 4% H_2O_2 in methanol followed by incubation with normal goat serum for 45 minutes. Excess serum was tapped off and the sections were incubated overnight at 4°C with rabbit anti-Rb antibody (C-15; M/S Santa Cruz, California) at 1:100 dilution (with 2% bovine serum and 0.1% Triton-X-100). After thorough rinsing in phosphate buffered saline (PBS) sections were incubated with 1:100 diluted biotinylated anti-rabbit antibody for 30 minutes followed by avidin-biotin complex (M/s Vector Laboratories, USA) at 1:100 dilution for 1 hour. A case of osteosarcoma was taken as a negative control. The antigen was localized using diaminobenzidine (DAB) as chromogen with 0.3% H_2O_2. Rb was evaluated as positive and negative nuclei. Tumors with >20% negatively stained nuclei were considered as showing altered protein expression.

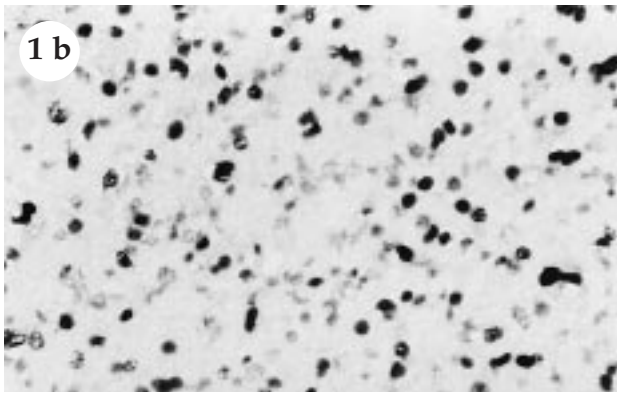
p53 – After rehydration the endogenous peroxidase was blocked with 2% H_2O_2 in methanol for 20 minutes followed by thorough PBS washing. The slides were then placed in staining trough kept in a 1 litre beaker containing preboiled 0.01 M citrate buffer (pH 6.0) and microwaved (BPL microwave oven, 700W), first for 10 minutes at 100% power and then for 5 minutes at 50% power and then cooled for 20 minutes. A thorough Tris buffered saline (TBS 0.05 M, pH 7.6) wash was followed

Table 1. **Summary of patients with p53 and pRb expression**

	A (n=16)	*GA (n=7)*	*AA# (n=19)*	*GBM (n=22)*
Age (Yrs.)				
Range	18–60	19–39	10–60	25–60
Mean	34.0	32.8	32.35	40.4
Sex (M:F)	7:1	5:2	12:7	15:7
Survival Status				
Well	12	4	10	5
Recurred	3	2	2	3
Expired	1	1*	3	3+11*
p53 positivity				
Range	0–56	13.6–70.6	0–77	12.3–85
Mean	17.9	41.8	32.14	42.3
pRb				
No.of cases positive	14	5	13	15
No.of cases negative	0	0	0	6
No.of cases+/–	2	2	6	1

A – Astrocytoma
AA – Anaplastic astrocytoma
GBM – Glioblastoma multiforme
* – Expired after recurrence
# – Lost to follow up – 4 patients
n – number of case





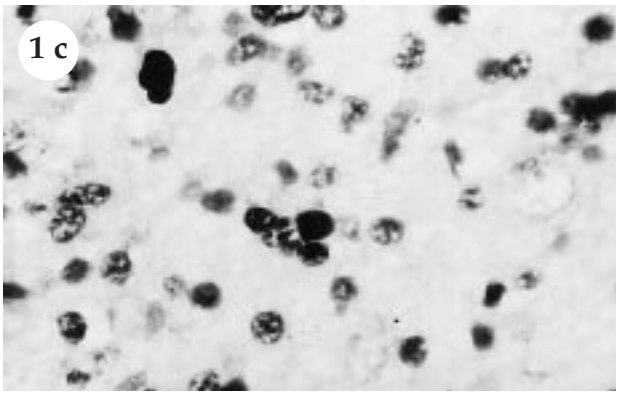


Figure 1. *Immunopositive pRb (c-15) nuclear labelling in a representative astrocytic tumor. (a) Most of the nuclei are pRb immunopositive (x400). (b) Heterogenous immunopositive pRb staining in a case of glioblastoma multiforme. Note the negative nuclei amidst the positive ones (x100). (c) Higher magnification (x400).*

by blocking of non-specific sites with normal horse serum (30 minutes). Excess of serum was drained from the sections, and antimouse p53 antibody (DO-1, M/s Santa Cruz, California) diluted 1:80 in TBS with 2% bovine serum and 0.1% Triton X-100 was applied and incubated overnight at 4°C. After thorough rinsing in TBS, section were then incubated with 1:100 diluted biotinylated anti-mouse secondary antibody for 45 minutes followed by avidin-biotin complex in 1:100 dilution (M/s Vector laboratories, USA) for 1 hour. A case of GBM which showed a very high positivity for p53 was taken as a positive control.

A negative control was run parallel with each batch by omitting primary antibodies. The antigen was localized using diaminobenzidine (DAB) as chromogen with 0.3% H_2O_2 in TBS and counterstained lightly with hematoxylin.

p53 reactive cells were examined and evaluated quantitatively. Endothelial cells and areas of tumor necrosis were excluded from evaluation. For each tumor, 1000 cells were counted and the p53 labelling was quantitated as percentage of labelled nuclei per 1000 cells.

Statistical analysis

The correlation between pRb, p53 and survival was analyzed using Kaplan Meier test. Fischer exact test was used to correlate pRb, p53 and tumor grade.

Results

The 64 astrocytic tumors chosen for the present study comprised of 16 astrocytomas 7 gemistocytic astrocytomas, 19 anaplastic astrocytomas and 22 glioblastoma multiforme (GBM). The diagnosis with patient characteristics is shown in *Tables 1*. The difference in survival time between astrocytoma and gemistocytic astrocytomas was not found to be statistically significant. However, statistically significant difference in the survival time was found between patients of astrocytomas vs those of anaplastic astrocytoma/glioblastoma (p=0.0135). The survival time for glioblastoma was much less.

Reaction patterns

pRb – The Rb protein was localized to the nucleus and its expression showed considerable variation. Forty seven out of 64 tumors (73.4%) expressed normal homogenous uniform Rb positivity in essentially all of the tumor cells *(Figure 1 a)*. Of these, 14 were astrocytomas, 5 gemistocytic astrocytomas, 13 anaplastic astrocytomas and 15 were GBM. Seventeen (26.5%) tumors showed abnormal heterogenous Rb staining pattern with an admixture of variable number of negative cells along with the positively stained cells. Of these, 2 each were astrocytomas and gemistocytic astrocytomas, 6 anaplastic astrocytomas and 7 were GBM.

The negative cells were present in small groups intimately admixed with the positive cells and were scattered throughout the tumor *(Figure 1 b, c)*. In one case of GBM expressing pRb heterogenously, a single tumor fragment was found to be completely devoid of pRb expression. Though heterogenous pRb expression appeared more common in GBM than in the other types of astrocytic tumors, statistically this was not significant and no correlation of altered pRb expression with tumor grade was found.

p53 – A strong immunostaining for p53 localised essentially to the nucleus was observed in 52 of 64 (81.2%) tumors (*Figure 2*). Of these, 10 were astrocytomas, 7 gemistocytic astrocytomas, 9 anaplastic astrocytomas and 20 GBM. The mean percentage of p53 positive cells were 17.9, 41.8, 32.2 and 42.3 for astrocytoma, gemistocytic astrocytoma, anaplastic astrocytoma and GBM respectively (*Table 2*). There was no statistically significant correlation of p53 positivity or the percentage of positive cells with the grade of the tumor.

Correlation of altered p53 and pRb expression

Overexpression of p53 protein was observed in 52/64 tumors (81.2%). Of these, 14 (27%) also showed altered pRb expression – one astrocytomas (7.1%), 2 gemistocytic astrocytomas (14.3%), 4 anaplastic astrocytomas (28.5%) and 7 GBM (50%). *(Table 2.)*

Apart from these 14, 3 tumors (one astrocytoma and 2 anaplastic astrocytomas) also expressed pRb heterogenously but these were negative for p53. The remaining 47 showed normal homogenous pRb expression. Most of the cases with heterogenous pRb expression were also positive for p53 and this was more common in GBM (50%).

Table 2. **Immunopositivity for p53 and pRb in 64 astrocytic tumors**

Cases	*p53+/pRb+ (%)*	*p53+/pRb± (%)*	*p53–/pRb+ (%)*	*p53–/pR± (%)*	*Total*
A	9 (56.3)	1 (6.2)	5 (31.25)	1 (6.25)	16
GA	5 (71.4)	2 (28.6)	0 (0)	–	7
AA	11 (57.9)	4 (21.0)	2 (10.5)	2 (10.5)	19
GBM	13 (59.1)	7 (31.9)	2 (9)	–	22
Total Cases	38 (59.4)	14 (21.9)	9 (14.0)	3 (4.7)	64

A – Astrocytoma
GA – Gemistocytic Astrocytoma
AA – Anaplastic Astrocytoma
GBM – Glioblastoma Multiforme

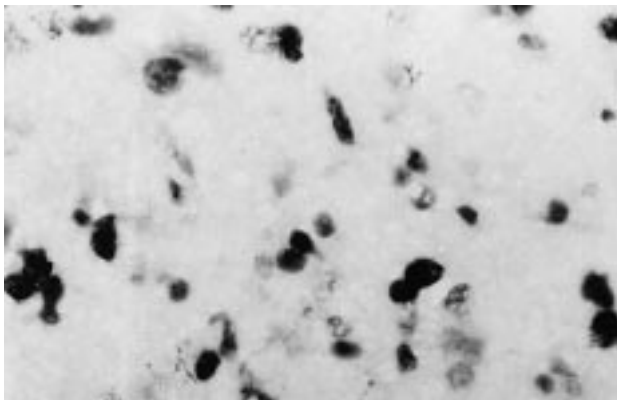


Figure 2. *Immunopositive p53 (DO-1) nuclear labelling in glioblastoma multiforme (x400).*

A fragment of tumor which was completely devoid of pRb expression showed a strong p53 positivity (100%). This tumor was heterogenous for pRb and also expressed mutant p53 protein.

Glioma grades with p53 and pRb expression

Of the 16 astrocytomas, 10 (62.5%) were positive for p53. One of them expressed pRb heterogenously. Six tumors were negative for p53 but expressed pRb homogenously except for one which expressed pRb heterogenously. Thus one tumor showed simultaneous abnormality of both p53 and pRb expression. *(Table 2.)*

All the 7 gemistocytic astrocytomas showed p53 positivity. Of these, 5 (71.4%) showed normal Rb positivity while 2 (28.5%) expressed pRb heterogenously. Thus, 2 of the 7 (28.6%) gemistocytic astrocytomas showed simultaneous p53 and pRb abnormality.

Of the 19 anaplastic astrocytoma, 15 (78.9%) were p53 positive. And of these 4 showed heterogenous Rb positivity, while in the remaining pRb was uniform. Two tumors which expressed pRb homogenously were also negative for p53. Out of the four tumors which were negative for p53, two expressed pRb homogenously and two heterogenously. Thus, 4/19 (21%) anaplastic astrocytomas showed simultaneous altered expression of p53 and pRb.

Out of 22 glioblastomas, 20 (91%) were p53 positive. Thirteen of these tumors expressed pRb homogenously while 7 (31.9%) showed heterogenous pRb positivity. Two (9%) tumors were negative for p53 and also showed normal pRb staining. Thus, 7/22 GBM (31.8%) tumors showed double abnormality of p53 and pRb expression.

Kruskal Wallis one way nonparametric analysis did not show any statistically significant difference between the different grades of tumor expressing both p53 and pRb (p=0.8344). However, 31% of GBM showed heterogenous pRb expression along with p53 overexpression as compared to 6 % of astrocytoma, 28.6% of gemistocytic astro-

cytoma and 21% of anaplastic astrocytoma. No statistically significant difference (p=0.6065) in survival rate could be found between the double positive cases with homogenous pRb staining and those with heterogenous pRb expression. The survival in GBM, appeared to be lower in patients expressing altered pRb, though statistically this was not significant.

Discussion

Our study on the expression of Rb and p53 protein included tumors from 64 patients classified according to the new WHO classification.[21] It follows several recent observations that suggest the involvement of these genes in the formation and progression of astrocytic tumors.[8,18]

Rb gene inactivation has been noted in astrocytic tumors by several investigators.[11,13,14,16,23,30] Hamel et al[14] studied the pRb expression in glioma cell lines using immunoprecipitation and reported that 8 of 24 lacked p110 Rb expression. Among the 17 primary brain tumors studied by them using Western blotting, 3 of the 10 WHO grade 3 and 4 gliomas showed loss of pRb expression. However, none of the WHO Grade 2 gliomas lacked pRb expression. Henson et al documented loss of heterozygosity (LOH) in 16 of 54 (30%) of high-grade astrocytomas, however LOH was not detected in 12 low grade gliomas.[14] When studied by immunohistochemistry, 3/9 cases with LOH and one tumor without LOH showed altered Rb protein expression. Nakamura et al[27] also reported altered pRb expression in 25/74 (34%) high grade astrocytoma using immunohistochemistry. Similar altered expression of the Rb protein was also observed in 26.5% of cases (17/64) with a portion of one GBM completely lacking pRb expression. Seven of these 17, were GBM (41.1%) indicating that pRb alteration is possibly more common in high grade gliomas. The definition of altered Rb protein reactivity differs greatly between the published reports. Some investigators consider that all nuclei in tumor cells should be positive[17] while others also include tumors with admixture of positively and negatively stained nuclei.[7] There are reports that only pRb is phosphorylated during G_1/S phase while unphosphorylated pRb which cannot be detected by immunohistochemical procedures[15] is found only in G_O and G_1 phase of the cell cycle.[3,26] At any given time the population of cells in G_o and G_1 phase is approximately 30–40% and considering that the half life of pRb in at least 12 hours,[3] we employed a 20% cut off for altered pRb. Hence, only tumors with more than 20% of negatively stained nuclei were considered as expressing altered pRb using this criteria.

There is substantial evidence implicating p53 in astrocytic tumors. Haapasalo et al[10] in a large series of astrocytic tumors (102 cases) detected positive p53 immunostaining in 49% of grade 3 and 4 tumors and 19 to 29% of grade II astrocytomas, but none in grade I astrocytomas. Aka et al[1] described LOH of the p53 gene in 11/16 (69%) cases of malignant astrocytoma of which 10 (91%) overexpressed p53 protein. Rasheed et al[28] reported p53 gene mutation in 17/120 gliomas with an increased incidence in patients with anaplastic astrocytomas. Iuzzolino et al[18] studied 52 low grade astrocytomas immunohistochemically and documented p53 overexpression in 32 of them (61.5%). Ellison et al,[8] using a panel of 5 antibodies to p53 noted detectable quantities of p53 in the cells of 3/16 diffuse astrocytomas, 8/14 anaplastic astrocytomas and 24/34 GBM. In our study 52/64 (81.2%) tumors were immunopositive for p53 protein which is in concurrence with the results of Aka et al.[1]

No association was observed between the p53 expression and patient survival in the present studies which is similar to the report by Rasheed et al.[28] However, our results vary from earlier reports in that there was no significant correlation of the p53 positivity or the percentage of p53 positive cells with the histological grade of tumor malignancy. Thus 62.5% of astrocytomas,100% of gemistocytic astrocytomas, 79% of anaplastic astrocytomas and 91% of GBM were p53 positive.

Recently Watanabe et al[31] reported p53 positivity in gemistocytic astrocytomas. Our study echos their findings in that 100% of the gemistocytic astrocytomas showed p53 positivity. This is of interest in view of the well documented progression of gemistocytic astrocytomas to GBM.

The present study highlights the simultaneous expression of p53 and Rb proteins in gliomas. In the English literature, there are only two reports, one by Lee et al[23] and another by Tsuzuki et al.[30] Lee et al documented LOH in 33 human gliomas on chromosome 10 and 17p in addition to Rb and p53 mutation using by RFLP-SSCP. They observed LOH on Rb in 54% of the gliomas with only 38% showing p53 mutation. Tsuzuki et al[30] studied alterations in the Rb, p53 and p16 genes in 23 astrocytic tumors, using PCR-SSCP followed by sequencing. They reported aberrations of the Rb gene in 3/23 cases (13%) and p53 gene mutations in 4/23 cases (17.4%), combined or simultaneous Rb and p53 aberrations were not noted in any case. However, in 2 cases, simultaneous point mutation of the p53 gene and deletion of the p16 gene was observed in their series. Unlike them, we found abnormal expression of both pRb and p53 in 14/64 cases (21.9%) immunohistochemically. It was also observed that Rb negativity along with p53 overexpression was more common in GBM (31.9%). This association was not present in any case of low grade astrocytoma. Our finding that p53 expression may co-exist with Rb loss, and implies some form of relationship between them.

The point in tumorigenesis at which a gene is implicated is an important issue and the immunostaining pattern for p53 and Rb may reflect this. For example, if all cells

lack Rb expression (or if there is a homogenous p53 positivity), it would suggest that the gene is related to tumor initiation. On the other hand, if Rb reactivity is heterogenous with admixture of positive and negative cells, a progressional role would be implied. Both of the above patterns of expression are noted in our present study. The identification of a complete pRb negative fragment in one of the tumors with homogenous p53 expression is in agreement with the alterations occurring early in the tumor transformation resulting in growth advantage. However, a heterogenous staining for pRb was observed in 17/64 tumors, which favours a progressional role for Rb in astrocytic tumor evolution. Since p53 staining was observed in majority of the tumors including low-grade astrocytomas with little difference in the percentage of positivity, an initiational role can be assigned to it but it may be of very little importance for progression.

The Rb and p53 genes control distinct regulatory pathways in the cell cycle, and are undoubtedly modulated at the gene or the protein level by the integration of various factors and intracellular signals. Both pathways are inactivated, either through mutation or as part of the tumor progression in astrocytic tumors. Though this is a preliminary study with small number of cases, there is no such report till date available in the English literature on the simultaneous expression of these two proteins in various grades of astrocytic tumors. The trend is promising in that a higher percentage of GBM cases showed simultaneous abnormality in both p53 and pRb expression. Detailed molecular studies are warranted in this direction to confirm this association. Further elucidation of the cell cycle regulatory pathways would help in classifying the role of Rb and p53 alterations in astrocytic tumorigenesis.

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