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Short Communication

Aberrant p53 Expression in Astrocytic Neoplasms of the Brain: Association with Proliferation

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Aberrant expression of the p53 suppressor gene was evaluated in 102 cases of astrocytic neoplasms. Immunohistochemical staining with a monoclonal antibody (DO-7) and a polyclonal antibody (CM-1) to p53 protein (both wild type and mutant) on formalin-fixed paraffin sections showed a strong correlation with malignancy grade. The staining was positive in 49% of malignant neoplasms (grades III and IV) and in 19 to 29% of grade II astrocytomas, whereas none of the grade I tumors were positive. p53 expression was significantly associated with proliferation rate determined by immunohistochemical proliferating cell nuclear antigen (PCNA)-staining (median PCNA-labeling index (%): 4.22 (DO-7-positive) versus 1.18 (DO-7-negative), P < 0.0001; 4.02 (CM-1-positive) versus 1.18 (CM-1-negative), P < 0.001). Interestingly, in the glioblastoma group (n = 44), p53-positive tumors had higher proliferation indices, suggesting that histologically similar tumors could be divided into prognostically different subgroups by immunohistochemical demonstration of aberrant p53 expression. (Am J Pathol 1993, 142:1347–1351)

Mutation of the p53 tumor suppressor gene is a widespread phenomenon in human malignancy, occurring, eg, in lymphomas, sarcomas, and in a variety of carcinomas.[1–2] Very recently, mutations of the p53 gene have also been described in series of human brain tumors.[2–6] Cytogenetic data and restriction fragment length polymorphism analysis (RFLP) indicate that loss of portions of the short arm of chromosome 17 also occurs in brain tumors, causing inactivation or loss of the remaining normal p53 gene allele. This suggests that stepwise genetic aberrations leading to loss of the wild type p53 protein may play a crucial role in the tumorigenesis of astrocytic brain tumors.

The p53 tumor suppressor protein is a nuclear phosphoprotein that normally functions as a negative regulator of cell growth. Point mutations of the p53 gene result in the formation of an abnormal mutant protein with a prolonged half-life and novel oncogenic properties. Accumulation of the mutated p53 protein can be detected by immunohistochemical staining of tissue sections.[7] Although preliminary, there is evidence that p53 mutation is associated with high-malignancy-potential type carcinomas characterized by rapid cell proliferation.[8] To our knowledge, no such data exist from brain tumors. Immunohistochemical analysis of p53 may have diagnostic and prognostic applications in many tumor types, including neoplasms of the brain. In this study, we report our results on aberrant p53 protein expression in a series of astrocytic neoplasms and compare the results with proliferative activity determined by immunohistochemical reactivity with a monoclonal antibody against the cell-cycle-related antigen (PCNA).

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Materials and Methods

The 102 astrocytic neoplasms for the study were obtained from consecutive operable brain tumor patients who had undergone surgery at the Tampere University Hospital, Tampere, Finland, between December 1987 and February 1992. The tumors had been classified and graded according to the World Health Organization nomenclature[9] and Burger et al,[10] also using glial fibrillary immunoacidic protein staining in differential diagnosis. Of the original 105 cases, three were excluded because of a small and/or necrotic specimen, leaving 102 tumors to be evaluated. These included 12 tumors of grade I, 21 tumors of grade II, 25 anaplastic astrocytomas (grade III), and 44 glioblastomas (grade IV). Thus, 68% of the tumors were malignant (grades III and IV). Six tumors showed additional oligodendrocytic differentiation (mixed oligoastrocytomas), and two of the 44 glioblastomas had an additional sarcomatous component (gliosarcomas). The remaining tumors were considered pure astrocytic neoplasms.

Immunohistochemistry

Representative paraffin blocks were chosen for all the immunohistochemical stainings. Four- to 5-μ-sections from routinely fixed (over 24 hours in neutral buffered formalin) paraffin-embedded blocks were cut and taken on Vectabond-treated slides (Vector Laboratories Inc., Burlingame, CA) and dried overnight at room temperature. The slides were dewaxed, rehydrated, and stained using a standard avidin-biotin-enhanced immunoperoxidase technique (Vectastain Elite kit, Vector Laboratories). A rabbit polyclonal antiserum CM-1[11] and a monoclonal antibody DO-7[12] to p53 (both purchased from Novocastra Laboratories, Newcastle, UK) were used at dilutions 1:1200 and 1:300, respectively, and incubated overnight at 4 C. Diaminobenzidine (0.03% in phosphate-buffered saline) was used as chromogen. Immunostaining with DO-7 was enhanced with silver intensification as described elsewhere.[13] The sections were counterstained with hematoxylin. Paraffin-pelleted cells of the T47d breast cancer cell line were used as positive control. To determine the p53 immunoreactivity in non-neoplastic human brain tissue, 14 neurosurgical samples from hemorrhagic, infectious, and gliotic brain were studied. Of these, only one arteriovenous malformation showed a few DO-7-positive astrocytic cells in gliotic tissue [<1/10 high-power fields (HPF)], whereas others were negative. Tumors with widespread, intense staining of neoplastic nuclei were considered positive, showing aberrant expression of p53. Samples in which only occasional nuclei (<1/10 HPF) exhibited weak positive staining for p53 were considered negative, ie, to represent the normal level of expression.

The method used for PCNA has recently been described by Siitonen et al.[14] Immunostaining was done as described for p53 with the mouse monoclonal antibody 19A2 (IgM subclass, Coulter Immunology, Hialeah, FL) at a concentration of 0.1 μg/ml. PCNA antigen immunoreactivity was enhanced by incubating tissue sections 10 minutes with 1:3 diluted target unmasking fluid (Sanbio, Uden, Netherlands) heated at 90 C in a microwave oven. Silver intensification was performed similarly as with $p53_{DO7}$. Proliferative normal tissues (gut, skin) were used as positive control.

The staining results were scored as a percentage of $PCNA_{19A2}$-immunoreactive cancer cell nuclei (PCNA labeling index, PCNA-LI) evaluated from 20 contigous high-power fields of a microscope equipped with a square lattice in the eyepiece. Necrotic or hemorrhagic areas were avoided. Faint, diffuse nuclear staining seen in some tissue sections was not recorded as positive for PCNA score. The counting was performed in an area of 20 lattices (0.56 mm^2 of neoplastic tissue). Simultaneously, using the same square lattice, the area covered by the nuclei was estimated by the point-counting method (volume percentage of nuclei, VPN). All evaluations were blind, ie, they were performed without knowledge of the clinical features, histopathological grading, or the clinical outcome of the patients.

Statistical Methods

The relationship between p53, histological grading, proliferation rate, and volume percentage of epithelium was assessed by the χ-square test, the Mann–Whitney test, and analysis of variance (ANOVA). The analyses were performed with Superior Performance Software System and Biomedical Data Processing software.

Results

Nuclear p53 immunopositivity was observed in 38 (DO-7) and 40 (CM-1) of the 102 tumors (37.2% and 39.2%, respectively). The correlation between the two staining methods was very good, as only eight of the 102 tumors were classified differently with DO-7 and CM-1 (Figure 1). Polyclonal $p53_{CM1}$

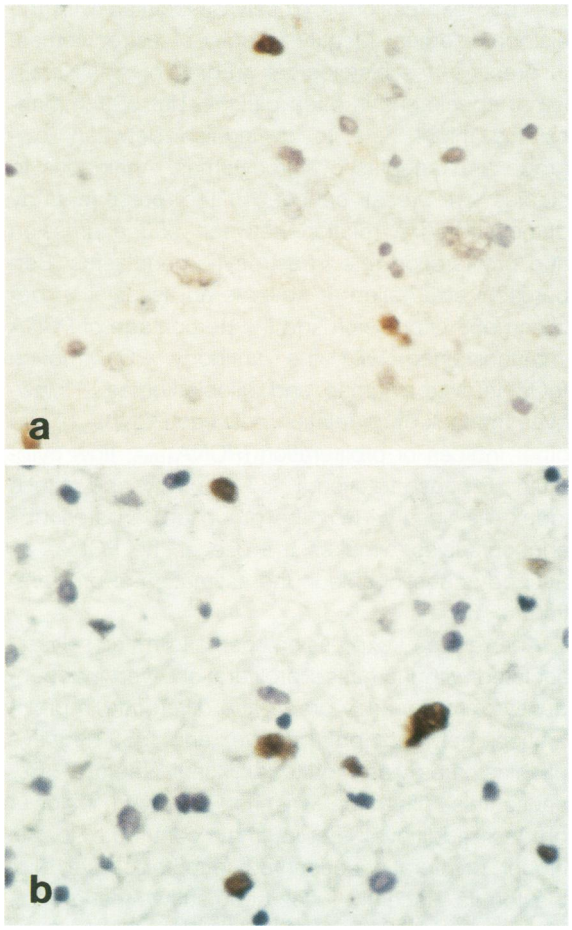

Figure 1. *Immunohistochemical staining of p53 in formalin-fixed, paraffin-embedded astrocytoma (grade II). Aberrant p53 protein nuclear labeling (brown) with polyclonal antiserum CM-1* **(A)** *and monoclonal antibody DO-7 to p53* **(B)**; ×320.

showed strong staining (ie, over 20% of nuclei positive) in 12 tumors and monoclonal $p53_{DO7}$ in six out of 102 tumors. Staining was not observed in non-neoplastic cells.

Patients' age was unrelated to p53 tumor protein status ($P = 0.17$, Table 1). The p53 expression was strongly associated with malignancy grade ($P = 0.0002$ [DO-7] and 0.0005 [CM-1]). None of the 12 grade I tumors were positive, whereas 39 to 60% of malignant astrocytic tumors (grades III and IV) were p53 immunopositive (CM-1 and DO-7). The volume percentage of nuclei (area covered by nuclei in the square lattice) is an estimate of cellularity, and it was highly associated with malignancy grade ($P < 0.0001$, $F = 15.35$, ANOVA), but had no significant association with aberrant p53 expression ($P = 0.09$ [DO-7], 0.3 [CM-1], Table 1).

ANOVA showed that the log-transformed PCNA-LI (according to the Kolmogorov–Smirnov test, transformation of the data was needed to achieve normal distribution for ANOVA) was strongly associated with malignancy grade ($P < 0.0001$, $F = 16.69$). Low-grade (I and II) astrocytic neoplasms showed a median PCNA-LI of 0.65% (mean ± SD 0.73 ± 0.69) and high-grade (III and IV) tumors a median value of 3.33% (4.41 ± 3.80, $P < 0.0001$, Mann–Whitney test). p53 expression was strongly associated with proliferative activity: p53-positive tumors had significantly higher median PCNA-LI than p53-negative tumors (DO-7: 4.22% versus 1.04%, $P < 0.0001$; CM-1: 4.02% versus 1.18%, $P = 0.0009$, Mann–Whitney test, Table 2). In the glioblastoma group, the difference of proliferation between $p53_{DO7}$-positive (median PCNA-LI 6.88) and negative tumors (median PCNA-LI 2.91) was highly significant ($P < 0.001$, Mann–Whitney test).

Discussion

Several groups have studied the frequency of loss of genetic material on the short arm of chromosome

Table 1. *Association of Aberrant p53 Expression with Characteristics of 102 Astrocytic Neoplasms*

	p53-negative	p53-positive	*P*
Patient age (mean±SD)			
DO-7	41 ± 20	46 ± 17	n.s.*
CM-1	41 ± 20	46 ± 17	n.s.*
Volume % of nuclei (VPN) (mean±SD)			
DO-7	25 ± 9	28 ± 10	n.s.*
CM-1	25 ± 9	27 ± 10	n.s.*
Histological grade [no. (%)]			
I DO-7	12 (100)	0 (0)	
CM-1	12 (100)	0 (0)	
II DO-7	17 (81)	4 (19)	
CM-1	15 (72)	6 (29)	
III DO-7	11 (44)	14 (56)	0.0002†
CM-1	10 (39)	15 (60)	0.0005†
IV DO-7	24 (55)	20 (46)	
CM-1	25 (57)	19 (43)	

* *t*-test; † χ square.

Table 2. *Association of Aberrant p53 Expression with Proliferation by Histological Grading (n = 102)*

	PCNA-labeling index (%)		
	p53-negative (mean±SD)	p53-positive (mean±SD)	*P**
All tumors			
DO-7	2.3±2.9	4.9±4.1	<0.0001
CM-1	2.4±2.9	4.5±4.1	<0.001
Histological grade			
I DO-7	0.6±0.6		
CM-1	0.6±0.6		
II DO-7	0.7±0.8	1.1±0.6	n.s.
CM-1	0.8±0.8	0.8±0.6	n.s.
III DO-7	3.1±4.0	2.8±2.0	n.s.
CM-1	2.1±2.5	3.4±3.2	n.s.
IV DO-7	3.8±3.1	7.1±4.3	<0.001
CM-1	4.3±3.5	6.5±4.4	0.07(n.s.)

* Mann–Whitney test.

17 in different grades of astrocytomas.[3–6] Restriction fragment length polymorphism analysis has shown that loss of heterozygosity on chromosome 17 occurs in various proportions in different grades of astrocytic neoplasms.[5,6] With single-strand conformation polymorphism assay, p53 mutations located on chromosome 17 have been demonstrated in 0 of 6, or 3 of 8 grade II astrocytomas,[3,6] in four of 14, one of three, or two of three anaplastic astrocytomas,[3,5,6] and in one of 10, or 16 of 37 glioblastomas.[3,5]

The same p53 mutation found in astrocytomas[4,6] have been also detected in the breast cancer line[15] that was used in the characterization of p53 antibodies (CM-1, DO-7) of the present study. These antibodies have been shown to permit sensitive detection of p53 by enzyme-linked immunosorbent assay and immunoblotting.[11,12] This indicates that the p53 proteins are similar and that the antibodies (CM-1, DO-7) permit sensitive detection of p53 also in astrocytic neoplasms.

Almost 40% of the astrocytic neoplasms in our material were immunoreactive to p53 protein. In a recent study, a monoclonal (PAb 1801) and a polyclonal antibody (CM-1) against p53 protein were used in brain tumors.[16] In that material, p53 protein expression was observed in 35 of 86 gliomas (grade I and II astrocytomas, eight of 21; anaplastic astrocytomas, six of 10; glioblastomas, 12 of 21). Our results are consistent with these findings, and the studies together show that the frequency of p53 mutations in different grades of astrocytic neoplasms as determined by immunohistochemistry is very similar to that determined by molecular genetic approaches.[3–6]

Supporting the theory that the p53 gene plays a central role in the control of cell transformation and proliferation, the present study showed that aberrant p53 expression is closely associated with the histopathological identity (grade) of astrocytomas. In practice, the histological grading of small brain tumor specimens is often difficult, but p53 immunostatus might help to distinguish between low-grade and anaplastic astrocytomas. Analogous to studies on breast cancer,[8,17] the present study demonstrated a relation between p53 expression and proliferation rate in astrocytic neoplasms. Barbareschi et al failed to observe an association between p53 expression and the percentage of PCNA immunostained with the antibody PC10-murine IgG2 in astrocytomas and glioblastomas.[16] However, the lack of correlation between $PCNA_{PC10}$ and other indices of proliferation ($PCNA_{19A2}$, flow cytometric S-phase fraction, Ki-67 reactivity, and mitotic index) as observed by the group of Barbareschi[18] and ourselves[14] makes the use of the $PCNA_{PC10}$ antibody questionable in the estimation of proliferation.

Aberrant p53 expression seems to be closely associated with increased proliferation even independently of malignancy grade: the difference in proliferation between p53-positive and p53-negative glioblastomas was highly significant (Table 2). Because this difference was not observed in lower grades, additional factors may contribute to the higher proliferation observed in p53-positive glioblastomas. Recently, it has been shown that a combination of loss of heterozygosity on chromosome 10 and p53 mutation occurs only in glioblastoma patients.[4] One possible explanation for the observed difference in proliferation might be the combined effect of mutations in p53 gene and putative tumor suppressor gene on chromosome 10.

The mutation of tumor suppressor gene p53 is widely involved in disordered growth control; it is not only a secondary consequence of the neoplastic phenotype.[19] According to D.P. Lane, normal p53 protein, guardian of the genome, accumulates after DNA damage and switches off replication, allowing extra time for repair of the genome. This arrest cannot be carried out in cells with p53 mutation or host/viral proteins binding to p53. Therefore, tumor cells with such changes accumulate mutations and chromosomal rearrangements, leading to rapid selection of malignant clones.[20] Supporting this view, our study showed that there is a strong relation between aberrant p53 expression and proliferation in astrocytic neoplasms, even in the same grade of malignancy (glioblastomas). This and the presence of p53 mutations in low-grade astrocytic tumors suggest that p53 mutations occur during the initial stages of tumor formation, perhaps allowing the transformed cells to proliferate. Whatever the

precise mechanism, immunohistochemical determination of p53 expression in routine paraffin-embedded material seems to be a useful tool in the diagnostic histopathological examination of brain tumors. Its strong correlation with the increasing malignancy grade makes it particularly helpful in grading and differential diagnosis of small brain tumor specimens. In addition, the strong correlation between grade, proliferation, and p53 expression suggests that p53 expression might have independent prognostic power in astrocytic neoplasms, as already shown in breast cancer,[8] and value in choosing the therapy of greatest benefit for the patient.

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