EXPRESSION OF p53 IN OLIGODENDROGLIOMAS

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SUMMARY

The expression of the nuclear protein p53 in oligodendrogliomas was investigated by immunohistochemistry, using a monoclonal anti-p53 antibody (DO-7) on formalin-fixed, paraffin-embedded material in 84 histologically verified cases, and compared with the histopathological grade and survival. p53-immunoreactive cells were found in 75 per cent of the samples acquired at the first biopsy. The p53 labelling index was not related to the degree of nuclear anaplasia. Tumour cases with more than 75 per cent p53 immunostained cells had a rapidly fatal clinical course. However, no significant correlation was found between p53 labelling index and tumour grade, mitotic index, or ploidy status. In most tumour recurrences ($n=25$), the p53 labelling index increased or remained at the level of the first biopsy. In five cases (6 per cent), p53 was absent in the first sample as well as in the recurrence. Irrespective of the underlying aberration of either the gene or the metabolic pathway of p53, it is concluded that a high percentage (i.e., more than 75 per cent) of p53-immunolabelled cells is predictive of an unfavourable clinical course, while a percentage lower than 75 per cent immunoreactive cells does not exclude a rapid fatal outcome.

KEY WORDS—Oligodendroglioma, immunohistochemistry, p53, tumour suppressor gene, mitotic index, DNA flow cytometry.

INTRODUCTION

When DNA of normal cells is damaged, the nuclear protein p53 is thought to accumulate and arrest the cell cycle at G1, presumably in order to enable repair of DNA damage.[1] If p53 is disrupted, genetically unstable clones could continue to divide, resulting in the accumulation of mutations and chromosomal rearrangements in subsequent generations.[2] While in non-neoplastic cells p53 expression is almost always below the level of immunohistochemical detection,[3] mutations in

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the p53 gene or aberrations in p53 degradation may result in elevated levels of the p53 protein, permitting its immunohistochemical detection. Although the connection between disruption of the p53 system and development of tumours remains speculative, p53 is immunohistochemically detectable in a wide variety of tumours.[4-20] In many,[4,5,8,9,12-14,16,18-20] though not all[6,7,10,15,17] human cancers, p53 immunoreactivity was found to be associated with morphological and cell proliferation-related tumour characteristics.

In astrocytomas, the number of p53 immunoreactive cells reportedly correlated well with malignancy grades.[21-24] Despite the demonstration of p53 mutations in oligodendrogliomas,[25] p53 immunoreactive cells have been recorded in only a few cases that have been investigated so far,[21,22,24] while no data relating p53 immunohistochemistry to tumour grade or clinical course are available.

The aim of this retrospective study was to investigate immunoreactivity for p53 in 84 cases and to

link the percentages of p53-positive cells with the histopathological grades and clinical courses. In addition, the data were correlated with the results of counting mitoses and DNA flow cytometry obtained in a previous study on the same group.[26]

MATERIALS AND METHODS

Clinical records

The clinical data and formalin-fixed, paraffin-embedded material of 111 patients with cerebral oligodendrogliomas were taken from the files of the University Hospital Rotterdam-Dijkzigt. In addition, tissues of ten patients with conditions leading to reactive gliosis were processed. The same samples of oligodendrogliomas have been used earlier in a retrospective study on the implications of DNA flow cytograms on the biological behaviour of oligodendrogliomas.[26] Adequate follow-up was obtained in 84 cases. The patients had been admitted to the hospital between 1972 and 1986. The age distribution at the first operation showed peaks around 35 and 55 years. Craniotomy was performed in order to decompress the brain or debulk the tumour. Patients who died within 2 weeks postoperatively were excluded from the study, since this short postoperative survival was considered as death related to surgical complications. Survival times were calculated from the date of the first craniotomy. All patients had died by the end of the study.

Grading the oligodendrogliomas and assessing the mitotic count

p53-immunohistochemistry was performed on slides adjacent to those used for histopathological grading, DNA flow cytometry, and counting mitoses in an earlier study.[26] The tumours were graded according to the scheme of Smith *et al.*,[27] taking endothelial proliferation, necrosis, nucleus to cytoplasm ratio, cell density, and pleomorphism into account. The variables were scored in a simple present–absent scheme, resulting in the attribution of four grades. The scheme has been proven to correlate significantly with survival.[27,28] The mitotic count was assessed by first selecting the highest cell density areas followed by randomly choosing fields, and then calculating the quotient of the number of mitoses and the total number of cells in the fields (objective × 400). The scores were grouped into three categories: a category of less than one mitosis; a category of more than five mitoses; and an intermediate group.

Immunohistochemistry

Immunohistochemistry was performed on 5 μm sections. The slides were deparaffinized and endogenous peroxidase activity was blocked with 3 per cent hydrogen peroxide (H_2O_2) in methanol, followed by preincubation with 5 per cent normal goat serum diluted in phosphate-buffered saline and bovine serum albumin (PBS–BSA) for 15 min at 37°C. All incubations were performed in a humidified chamber. The primary antibody was mouse anti-p53 (DO-7), diluted 1:25 in PBS overnight at 4°C. DO-7 (Dako, Denmark) was raised against recombinant human wild-type p53 protein expressed in *Escherichia coli*. This antibody recognizes an epitope in the N-terminus of the human p53 protein, residing between amino acids 35 and 45. Rinsing the excess antibodies or conjugates was done by three 5 min washes in PBS. The avidin–biotin–peroxidase complex method was used as the detection system. Incubation with the biotinylated goat anti-mouse immunoglobulin (Dako, Denmark) diluted 1:400 was followed by incubation with avidin and biotinylated peroxidase complexes (Dako, Denmark) for 30 min at room temperature. Final visualization was achieved by incubation with 0·02 per cent diaminobenzidine (DAB) in PBS and 0·075 per cent H_2O_2 for 7 min in darkness. As a control, the primary antibody was replaced by PBS. The slides were counterstained with haematoxylin.

Scoring p53-positive cells

The percentage of p53-positive cells was established by the quotient of the number of p53-positive cells and the total number of cells per ten fields × 400 magnification using a grid. The counts were repeated. The p53 labelling index (LI) was defined as 100 × number of labelled nuclei ÷ total number of nuclei counted. Five groups were distinguished: a group without positive cells and four groups with increasing LI (i.e., 1–25 per cent; 26–50 per cent; 51–75 per cent; more than 75 per cent).

Statistics

The statistical tests were performed using the Statistical Package for the Social Sciences (SPSSX package). Since the frequency distribution of the survival times of the patients was exponential, e-log transformation was performed in order to allow the use of parametric tests. Analysis of variance (ANOVA) was used for the detection of
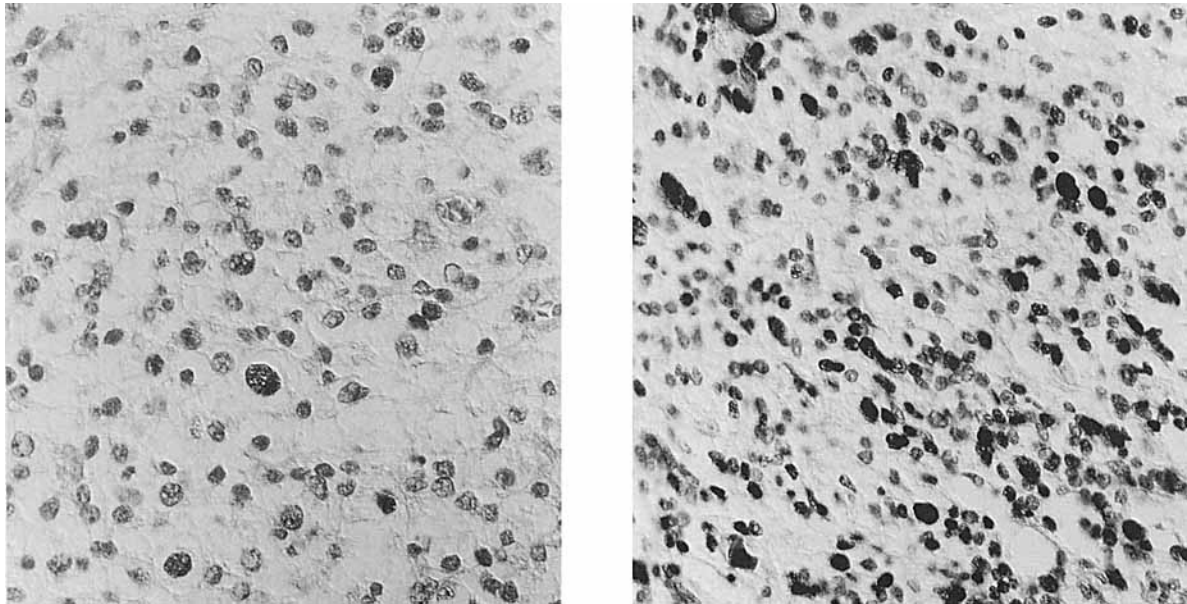
Fig. 1—(a) Oligodendroglioma grade B, stained for p53. Clonal patterns of p53-positive cells were not distinguished; p53-positive cells were always intermingled with p53-negative cells. (b) Oligodendroglioma grade C, stained for p53. p53 immunoreactivity was independent of the degree of nuclear anaplasia

the main effects of the grading scheme and the p53 LI. *A posteriori* testing was done using the Student–Newman–Keuls test with significance at the 0·05 level. Dependences between the results of p53 immunostaining and those of histopathological grading, DNA flow cytometry, and counting mitoses were tested by calculating Cramér's contingency coefficient.

RESULTS

Control sections of normal brain (grey and white matter) and sections of reactive gliosis did not contain p53-positive cells. The positive nuclei showed a granular staining pattern. Staining was always confined to the nucleus and was not seen in the cytoplasm of the neoplastic cells (Figs 1a and 1b). Endothelial cells were always negative for p53, even when endothelial proliferation was present.

Seventy-five per cent of the oligodendrogliomas contained p53-positive cells, while 25 per cent of the tumours did not have any positive cells (Table I). The inter-observer variability was about 5 per cent; the intra-observer variability remained below 2 per cent. The mean survival times for the five

Table I—Survival times for p53 labelling index (LI)

p53 LI	n		Survival time (months)		
			Mean	SD	SEM
0%	21	(25%)	34·95	30·48	6·66
1–25%	23	(27%)	52·83	59·81	12·46
26–50%	23	(27%)	29·61	30·69	6·39
51–75%	10	(12%)	35·60	28·81	9·12
>75%	7	(8%)	9·29	2·93	1·11
	84	(100%)			

n=number of patients; mean=mean survival time; SD=standard deviation; SEM=standard error of the mean.

groups based on p53 LI are listed in Table I, and the corresponding survival curves are plotted in Fig. 2. The curve of the highest p53 LI (i.e., >75 per cent) falls the fastest, and a significant difference between this curve and the curves of tumours with a p53 LI of less than 75 per cent was found ($P<0·05$)(Fig. 2). The mean survival times for the four groups distinguished by the grading system are listed in Table II. An increase in tumour grade
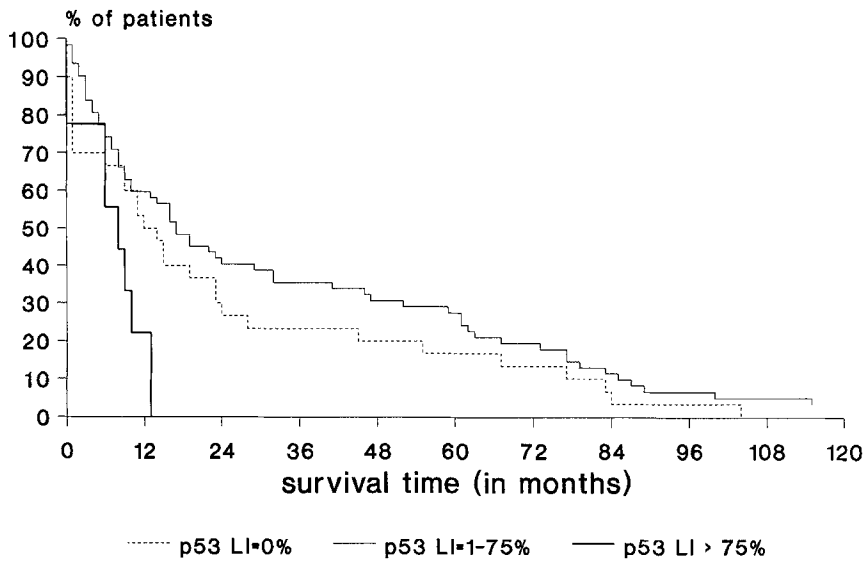
Fig. 2—Survival curves according to the percentages of p53-positive cells. The curve for p53 LI between 75 and 100 per cent is clearly separated from the curves for all distinct LI groups lower than 75 per cent. Only one high-grade tumour (grade D) was found among the seven samples in the >75 per cent curve (Table III)

Table II—Survival times for histopathological grades

Histopathological grades	n	Survival time (months)		
		Mean	SD	SEM
A	22 (26%)	56.36	59·98	12·79
B	41 (49%)	32·77	31·50	4·92
C	6 (7%)	31·33	30·71	12·53
D	15 (18%)	17·20	19·72	5·10
	84 (100%)			

See footnote to Table I.

Table III—Cross-table histopathological grades versus p53 LI

p53 LI	Histopathological grade				Total
	A	B	C	D	
0	11%	14%	0	4%	29%
1–25%	6%	9%	3%	9%	27%
26–50%	5%	14%	1%	5%	25%
51–75%	2%	5%	2%	2%	11%
>75%	0	5%	1%	1%	7%
Total	24%	47%	7%	21%	100%

Cramér's contingency coefficient=0·444; P=0·194.

parallels a decrease in mean survival. The survival rates for the two intermediate grades (i.e., grades B and C) are virtually indistinguishable. ANOVA showed a main effect of the grading system on the survival rates (P=0·035). *A posteriori* testing (Student–Newman–Keuls) revealed a significant difference between grades A and D at the 0·05 level.

Table III represents the crosstable between p53 LI and histopathological grade. No correlation between the results of p53 immunostaining and histopathological grading was found (Cramér's contingency coefficient=0·444; P=0·194).

Thirty-one per cent of the oligodendrogliomas were DNA diploid, 39 per cent had a DNA-tetraploid pattern, while 31 per cent were aneuploid.[26] Although the mitotic count correlated with the survival time (P<0·0038), the ploidy status did not.[26] No dependence between the ploidy status of the oligodendrogliomas and the p53 LI (Cramér's contingency coefficient=0·323; P=0·527) was found.

In 26 per cent of the tumours, no mitoses within 3 HPF (high-power fields) were seen. In 57 per cent the mitotic count was between 1 and 5, while in 17

Table IV—Cross-table p53 LI in successive biopsies ($n=25$)

p53 expression in recurrence	p53 expression in primary tumour				
	0	1–25%	26–50%	51–75%	>75%
0	5	0	1	0	0
1–25%	1	4	2	2	0
26–50%	0	0	1	0	0
51–75%	1	2	2	0	0
>75%	0	1	2	1	0

In ten cases the p53 LI was equal in both biopsies, wheras in another ten cases the level of demonstrable p53 had increased. In five cases the p53 LI in the recurrence was lower than that in the initial biopsy, most likely caused by sampling errors. Only in two cases was expression of p53 found in the recurrence, while the primary tumour was immunonegative. In five cases, p53 was demonstrable neither in the primary tumour, nor in the recurrence.

per cent more than five mitoses were counted.[26] No dependence between the mitotic index and the p53 LI was found (Cramér's contingency coefficient$=0.282$; $P=0.510$).

Table IV compares the p53 LI of the first biopsy with that of the second biopsy in the 25 patients who underwent craniotomy for recurrent tumour. In ten cases, no change in the p53 LI was observed in the two successive biopsies. In ten cases, an increase in p53 expression in the recurrent specimen was seen. In five cases, the p53 LI was lower in the second specimen. In two cases, the absence of p53 in the primary biopsy was followed by demonstrable p53 in the recurrence. In five cases, p53 was found in neither the first nor the second sample (Table IV).

DISCUSSION

The present finding of p53-immunolabelled cells in the majority of oligodendrogliomas is compatible with the finding of mutations in the p53 gene in these tumours.[25] The low percentages of p53 immunoreactive cells encountered in oligodendrogliomas reported previously might be explained by the small numbers of tumours investigated.[21,22,24,29] There are, however, some pitfalls in the interpretation of the results of p53 immunohistochemistry.[30] Negative immunostaining in the presence of a mutation might be the result of gross deletion abolishing all p53 production or, alternatively, may imply that the protein product of the

mutated gene has remained unstable, and thus undetectable by immunohistochemistry. Positive p53 immunohistochemistry without a mutation present may be due to an interruption of the normal degradative pathway of p53. In a recent paper on 34 astrocytomas, comparing the results of p53 immunohistochemistry with those of single-strand conformational polymorphism (SSCP), cases of either false-negative or false-positive immunohistochemistry were shown.[31] Despite possible discrepancies between the findings at the DNA level, on the one hand, and at the protein level, on the other, p53 immunohistochemistry has value for monitoring the functional status of the protein per se.

An important finding of this study is that high expression of p53 heralds a rapidly fatal clinical course. However, low expression of p53 does not correspondingly predict a favourable outcome. Although an increased percentage of p53-positive cells was found in the majority of the recurrences (Tables IV), no correlation between p53 expression and tumour grade was revealed. In four studies mainly concerning astrocytomas, the p53 LI reportedly matched histopathological grades.[21–24] In most,[32–35] but not all,[36] studies on glial tumours, mutations in the p53 gene were associated with tumour grade or tumour progress. There is a lack of correlation between p53 LI and tumour grade in oligodendrogliomas, possibly because of defective p53 genes present in low-grade tumours, while in high-grade oligodendrogliomas both alleles for the gene might have been lost, resulting in negative immunostaining. Alternatively, subsets of oligodendrogliomas with genetic defects without involvement of p53 might exist: in five cases, p53 was not found in the primary tumour nor in the recurrence. Still, high expression of p53 is predictive of short survival, irrespective of tumour grade.

Although in various epithelial neoplasms the p53 LI has been linked with aneuploidy,[5,8,19,20] no similar relation was shown in the present study. In neoplasms of the lung, the expression of p53 varied through the cell cycle and increased with the percentage of cells in the S-phase fraction calculated from DNA flow cytograms.[11] Controversial results concerning p53 expression and proliferation markers in glial tumours exist in the literature, while data specifically addressing oligodendrogliomas are missing. Jaros et al. found a relation between the expression of p53 and the Ki-67 labelling index in adjacent slides of 43 astrocytomas,[21] roughly indicating a link between proliferation fraction and p53 expression. However,

no such correlation was found in a study including 46 astrocytomas, in which antibodies against proliferating cell nuclear antigen (PCNA) were used.[29] The mitotic index did not correlate with the p53 LI in the oligodendrogliomas studied here. Double-labelling techniques using proliferation markers in combination with p53 antibody might disclose a relation between the expression of mutant p53 and the cell cycle phase at the level of the individual cell.

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