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Original Paper

Demonstration of p53 Protein and *TP53* Gene Mutations in Oligodendrogliomas

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Paraffin embedded tissue of 84 oligodendrogliomas (63 primary tumours, 21 recurrences), 21 glioblastomas with oligodendroglial growth pattern (15 primaries, 6 recurrences) and 17 mixed gliomas was investigated for the presence of mutations in exons 5–9 by means of single stranded conformation polymorphism (SCCP), temperature gradient gel electrophoresis (TGGE) and direct DNA sequencing. In parallel, p53 protein accumulation was determined by means of immunohistochemistry. The percentage of mutations was found to be higher than previously reported (6 of 44 grade II oligodendrogliomas, 4 of 19 grade III oligodendrogliomas, 4 of 15 glioblastomas). In 4 cases, the mutations lead to distinct changes in the primary or secondary structure of the protein (cysteine → tyrosine, proline → leucine) and were associated with marked accumulation of p53 protein. A significant correlation between p53 protein accumulation and *TP53* gene aberrations was found ($P < 0.001$), although p53 protein accumulation was detected more often than *TP53* gene anomalies, indicating that factors other than *TP53* gene mutation may also lead to a p53 protein accumulation in the tumour cells. A significant correlation was found for p53 protein accumulation and tumour grade but not *TP53* gene mutations. In conclusion, evaluation of p53 protein accumulation reflected the clinical course of oligodendrogliomas better than the mere presence of *TP53* gene mutations.

Key words: oligodendrogliomas, p53 protein accumulation, *TP53* gene mutation

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INTRODUCTION

THE p53 product is a nuclear phosphoprotein with a molecular weight of 53 kD involved in critical pathways of cellular growth and differentiation, including arrest in the G1 phase of the cell cycle, initiation of DNA repair, and induction of apoptosis [1]. Defects in the *TP53* gene, which is located on the short arm of chromosome 17, may lead to an impairment of the tumour suppressive functions of the protein. Production of aberrant p53 protein may lead to an accumulation of the protein in the cell, permitting its immunohistochemical detection.

Elevated levels of the p53 protein and the presence of *TP53* gene mutations have been shown for a large variety of tumours [1]. In the most frequently studied tumour groups of primary brain tumours, astrocytomas and glioblastomas, *TP53* gene mutations have been found in up to 46% [2]. Although only 12% of oligodendrogliomas have been found to harbour a *TP53* gene mutation [3], elevated levels of the p53 protein have been reported in 75% of oligodendrogliomas in another study [4].

The present study focused on the differences previously reported for the mutation rate of the *TP53* gene [3] and the expression of the p53 protein [4] in oligodendrogliomas. A demonstration of a discordance in the *TP53* gene mutation rate and the p53 protein accumulation in the same tumour collective would suggest that other cellular regulators might also be involved in the accumulation of p53 protein in tumour cells.

PATIENTS AND METHODS

Selection of cases

Cases between 1983 and 1993 were selected from the files and reviewed by two neuropathologists for confirmation

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of the original diagnoses and tumour grading according to WHO [5]. 59 oligodendrogliomas grade II (44 primaries, 15 relapses), 25 oligodendrogliomas grade III (19 primaries, 6 relapses), 21 glioblastomas with oligodendroglial growth pattern (grade IV, 15 primaries, 6 relapses), and 17 mixed gliomas (II/III, primaries) were chosen for further analysis. Patients with oligodendrogliomas consisted of 34 female and 44 male patients (mean age 47.5 years and 43.7 years, respectively), and those with mixed gliomas consisted of 4 female and 13 male patients (mean age 40 years and 39.2 years, respectively).

Preparation of DNA

DNA was extracted from paraffin embedded tumour specimens according to Kiene and associates [6]. Briefly, 8 μm sections were cut and boiled for 20 min in 200 μl sterile water in the presence of 50 μl chelating resin (Bio Rad Chelex 100, CAS 68954-42-7). The tubes were centrifuged for 10 min at 14 000*g* in a microfuge and the aqueous phase containing the DNA was recovered for amplification of *TP53* exons by polymerase chain reaction (PCR). Alternatively, DNA was extracted from 2–3 8 μm sections using the Cleanmix purification system (Talent srl, Trieste, Italy) according to the manufacturer's protocol. The latter method resulted in more consistent yields and better quality of DNA.

PCR amplification of TP53 *exons*

Exons 5–9 of the *TP53* gene, together with their neighbouring intron splice sites, were amplified by PCR using primers previously described [7]. The presence of DNA was evaluated in an initial PCR using the primer pair for amplification of exons 8 and 9. Each 100 μl reaction mix contained 3–10 μl of genomic DNA preparation, oligonucleotide primer (final concentration 200 nM), nucleotides (final concentration 200 nM), 10 μl of 10 × Taq buffer, magnesium chloride (final concentration 2 mM), and 5 U of Taq polymerase (GIBCO, 80388A). After denaturation of DNA for 10 min at 94°C, PCR was conducted for 1 min at each of the temperatures 57°C, 72°C, and 94°C for 40 cycles. A final 2 min annealing step followed by 10 min for chain extension ended the programme. The presence of the PCR product was confirmed by agarose gel electrophoresis of 9 μl of the reaction mix.

Single stranded conformation polymorphism (SCCP) screening for TP53 *exon mutations*

Depending on the DNA content of the PCR product, 2–15 μl of the PCR product was boiled for 10 min in the presence of formamide (USB cat.70724). The mixture was chilled on ice before electrophoresis in a 6% acrylamide Tris-borate/EDTA buffered gel. Electrophoresis was performed at 30–50 W in a cool room at 4°C. All PCR products of exons 5, 7, 8 and 9 were electrophoresed at least once without glycerol, and products of exon 6 with 5% glycerol. The bands were silver stained according to the manufacturer's modified silver stain protocol (Bio Rad cat. 161-0443). In cases where no mutation was found, the SSCP was repeated twice, with 5% and 10% glycerol.

Temperature gradient gel electrophoresis (TGGE)

Exon 6 mutations detected by SSCP analysis were additionally confirmed by TGGE. Fifty microlitres of PCR product was precipitated with ammonium acetate/ethanol. DNA was eluted in 10–30 μl of MOPS buffer depending on the DNA content of the sample previously determined by electrophoresis of 9 μl of PCR product. TGGE was performed as previously reported [8]. Wild type controls of exon 6 were evaluated in parallel.

DNA sequencing

Prior to sequencing, DNA was purified anew from the paraffin embedded tissue to guarantee that the sequence analysis yielded results independent of those from SSCP. PCR product was purified with the Cleanmix purification system (Talent srl). Cycle sequencing was performed with the Sequitherm cycle sequencing kit (Epicentre Technologies, Madison, Wisconsin, U.S.A.) according to the manufacturer's protocol using ^{32}P end-labelled primers. The *TP53* exons were sequenced from both the 3'-end and 5'-end using the same primers as for PCR amplification of *TP53* exons. Alternatively, a second 5'-end exon 6 primer [8] was used which hybridised to an intron sequence located nearer to the exon 6. Wild type controls were included for all exons.

Immunohistochemical detection of p53 protein

Wild and mutant types of the p53 protein were detected in paraffin embedded sections using monoclonal anti-p53 antibodies (DAKO M 7001, clone DO-7, dilution 1:50) according to the manufacturer's specifications. Biotinylated secondary antibody (Sigma B7264, dilution 1:60) and peroxidase-labelled streptavidin (DAKO K377, dilution 1:100) were added in sequence. Diaminobenzidine (Sigma D5637) was used as chromogen. All negative stains were repeated at least once.

Determination of the p53 labelling index

The p53 labelling index (p53 LI) was evaluated as previously described for assessment of the proliferation index [9]. Unlabelled and labelled cells were counted in one high power field (0.031 mm^2) in the tumour area showing the highest density of labelled cells. The choice of the tumour area thus resembled the tumour grading which is established from the most dedifferentiated area of the tumour.

Statistical analysis

All statistical analyses were calculated using the SSPS for Windows software release 6.0. Statistical analyses were computed separately for groups of primary oligodendrogliomas ($n = 78$), and mixed gliomas ($n = 17$), and for a longitudinal study of oligodendroglioma recurrences ($n = 27$).

RESULTS

Immunohistochemical demonstration of p53 protein accumulation

In grade II oligodendrogliomas, p53 protein was detected in significantly fewer cases than in grade III oligodendrogliomas and glioblastomas ($P < 0.001$, Table 1, Figure 1). The p53 LI correlated significantly with the tumour grading (Table 1, $P < 0.05$ for comparison of grade II versus grade III oligodendrogliomas, $P < 0.02$ for oligodendrogliomas grade II versus oligodendrogliomas III and glioblastomas).

Table 1. p53 accumulation in all oligodendrogliomas, glioblastomas and mixed gliomas

Number of cases	Tumour	Grade	Number of cases with p53 LI>0	Mean LI (%) ± S.E.M.
52*	Oligodendroglioma	II	6	1.9 ± 1.2
26*	Oligodendroglioma	III	14	13.1 ± 4.7
24*	Glioblastoma	IV	14	23.1 ± 6.6
9	Mixed glioma	II	6	10.3 ± 5.2
8	Mixed glioma	III	5	16.0 ± 7.3

*Note that in three recurrences, p53 LIs could not be evaluated due to lack of tumour issue; thus the total number of cases is 102, and not 105. The shift in the numbers of cases between the group of oligodendrogliomas and glioblastomas is due to changes in grading in the recurrences of oligodendrogliomas.

LIs > 10% occurred almost exclusively in malignant tumours (13 of 78 cases, Figure 2). In addition, the mean interval between first presentation and recurrence of the tumour was 18.6 months for LIs < 10% compared with 35.6 months for LIs > 10%, but this difference was non-significant.

In the group of mixed gliomas, p53 protein was detected in 6 out of 9 grade II tumours (mean p53 LI = 10.3%) compared with 5 out of 8 grade III tumours (p53 LI = 16%). Differences were non-significant.

Comparing primary oligodendrogliomas with recurrences, no significant differences were found in the number of cases with p53 accumulation nor in the p53 LI.

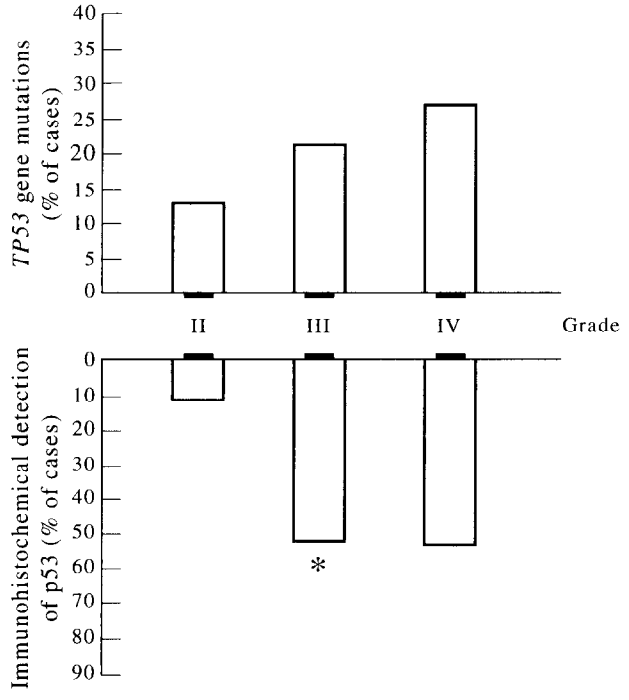

Figure 1. Relative frequencies of *TP53* gene mutations and p53 protein accumulation versus tumour grading in oligodendrogliomas and glioblastomas with oligodendroglial growth pattern (primary tumours = 78). *$P < 0.001$.

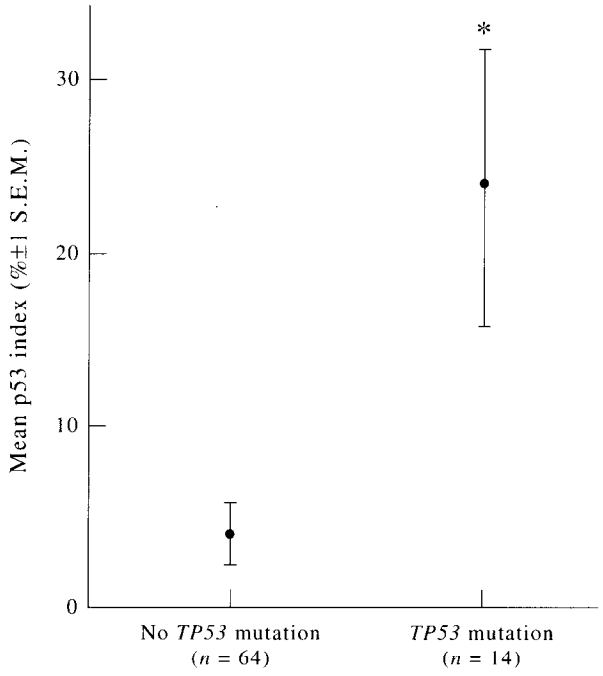

Figure 2. p53 protein labelling indices in primary oligodendrogliomas. Dermination of p53 LI in one high power field (0.031 mm^2) in the tumour area with the highest number of labelled cells (primary tumours = 78). *$P < 0.001$.

Detection of TP53 *mutations*

Initial SSCP screening for *TP53* mutations of all oligodendrogliomas and grade IV gliomas (primaries and recurrences, $n = 105$) revealed *TP53* mutations in 16 patients. In 4 of these, mutations were only detected in the primary tumour but not in the tumour recurrence. The presence of mutations in those 4 questionable negative recurrences were subsequently confirmed by direct DNA sequencing. Since 10 of the mutations were located in exon 6, for which only few mutations have been reported in the literature [10], mutated cases of exon 6 were additionally assessed with TGGE. In one of the exon 6 samples positive for a mutation in SSCP, the mutation was not confirmed by TGGE. DNA sequence analysis further revealed one case to be wild type *TP53* exon 7 which was thought to harbour a mutation according to SSCP analysis. DNA sequencing yielded readable sequences in 11 of the 14 mutated samples (Table 2).

The p53 LI correlated highly significantly with the presence of *TP53* gene mutations ($P < 0.001$, Table 1), with cases with *TP53* mutations having a significantly higher p53 LI than those without *TP53* mutations ($P < 0.001$, Figure 3). Despite this correlation, there was a discordance between presence of mutations and detectable levels of p53 protein in 22 cases. In 15 of these cases, p53 protein was detected but there was no mutation, and in 7 cases mutations did not lead to p53 protein accumulation (Table 2). None of these 7 cases was a silent mutation. Interestingly, in 6 of these cases the mutation was located in exon 6. Very high p53 LIs were found in the remaining 5 cases (cases 2, 7, 10, 13 and 14) for which sequencing data were available. 4 of these cases harboured mutations, most likely leading to distinct changes either in the primary structure of the protein (case 10, codon 190; case 14, codon 151; Table 3), with a change of proline to leucine or a change in the secondary structure, with a mutation from a tyrosine to

Table 2. Correlation matrix of tumour grading, p53 LI, TP53 *gene mutations, age, gender and interval between first operation and tumour recurrence in oligodendrogliomas*

	p53 labelling index	*TP53* mutation	Age	Gender	Interval
Grading	$r = 0.28$	$r = 0.14$	$r = 0.27$	$r = -0.05$	$r = -0.45$
	$P < 0.02$	n.s.	$P < 0.02$	n.s.	$P < 0.05$
p53 labelling index		$r = 0.39$	$r = -0.14$	$r = -0.003$	$r = -0.19$
		$P < 0.001$	n.s.	n.s.	n.s.
TP53 mutation			$r = -0.06$	$r = 0.03$	$r = -0.22$
			n.s.	n.s.	n.s.
Age				$r = -0.16$	$r = -0.28$
				n.s.	n.s.
Gender					$r = -0.21$
					n.s.

n.s., not significant. All significance levels were calculated two-tailed, $n = 78$, $n = 27$ for variable 'interval'.

cysteine (case 2, codon 220, Figure 4) or cysteine to tyrosine (case 7, codon 176). In the group of mixed gliomas, no significant correlation was found between p53 LI and p53 mutations.

Age, interval between first presentation and tumour recurrence and grade

The age of the patients correlated significantly with tumour grading ($P < 0.02$, Table 1), as did the time interval between first tumour operation and tumour recurrence ($P < 0.05$, Table 1) with tumour grading.

DISCUSSION

p53 protein accumulation in oligodendrogliomas has previously been investigated in detail only by Kros and colleagues [4] who reported increased levels of p53 protein in 63 of 84 oligodendrogliomas. In the present study the percentage of cases with p53 protein accumulation was less than half of that reported [4] (30% versus 75%). Differences in the composition of the tumour samples in the two studies could be one reason for this discrepancy, whereas methodological differences are unlikely to be the cause since the same anti-p53 antibody clone was used and all negative stains were repeated at least once. Unlike our study, Kros and coworkers did not find a significant correlation between histological grade and p53 protein accumulation ($P = 0.194$). This might possibly be due to differences in the assessment of the p53 LI in the two studies. In the present study, the p53 LI was evaluated from those areas with the highest number of labelled cells whereas Kros and colleagues [4] did not specify which areas of the tumour were chosen for measurement of p53 LI.

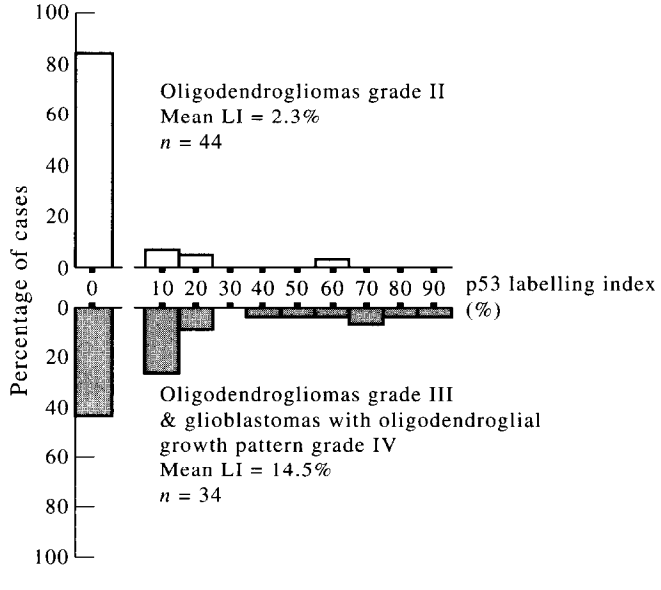

Figure 3. Mean p53 protein labelling indices in oligodendrogliomas with and without *TP53* gene mutations (primary tumours = 78).

In the report of Kros and colleagues [4] and in studies on astrocytic tumours [11] a significant correlation between p53 LI and prognosis has not been found. Kros and colleagues [4] reported a limit for p53 LIs of > 75% (7/84 cases) for a rapidly fatal clinical course, although this finding was statistically non-significant. In the present study, those with a p53 LI > 10% were found to develop a recurrence faster than those with a p53 LI < 10%, but this difference was also non-significant.

Data on *TP53* gene mutations in oligodendrogliomas are still sparse. Whereas over 300 cases of astrocytomas have been screened for *TP53* gene mutations, Bögler and colleagues [10] quote only 67 cases of oligodendrogliomas in their review of *TP53* mutations in human brain tumours. The frequency of *TP53* mutations in oligodendrogliomas grades II and III has previously been reported as 12%. In the present study, the mutation rate of oligodendrogliomas was found to be higher (6 of 44 cases, 13.6% in grade II oligodendrogliomas, 4 of 19 cases, 21% in grade III oligodendrogliomas). Nevertheless, the number of cases is still small, thus there was no statistically significant difference between the mutation rates reported here and those reported in the literature. In glioblastomas with oligodendroglial growth pattern, the mutation rate was lower than that previously reported for glioblastoma multiforme (4 of 15 cases, 26.7%) [10], but again this difference was statistically non-significant.

With regard to the location of mutations, interestingly, 10 of 14 mutations were located in exon 6 (Table 2). None of the mutated codons have been described in a glioma before, although most of the mutations have previously been detected in other human tumours. The C-T transition in codon 190 has been found in colon carcinoma and leukaemia; the G-T tranversion in codon 202 has been described in a case of leukaemia; an A-G transition has been detected in codon 210 in a hepatocellular carcinoma, but not the A-C transversion found in another case in this position. The A-G transition in codon 220 is very frequent in a variety of

Table 3. TP53 *gene mutations in oligodendrogliomas and mixed gliomas*

Case	Age	Gender	Grade	Exon	Codon	Base change	p53 LI	Interval*
Oligodendrogliomas								
1	32	m	II	6	210	AAC → GAC	0	42
2	25	m	II	6	220	TAT → TGT	60	27
3	29	m	II	6	201	Deletion of T	0	49
4	28	m	II	6	210	AAC → CAC	0	n.r.
5	57	m	II	6	n.d.	n.d.	0	n.r.
6	45	m	II	7	239	AAC → GAC	0	n.r.
7	35	m	III	5	176	TGC → TAC	67	35
8	31	f	III	8–9	n.d.	n.d.	82	n.r.
9	57	f	III	6	202	CGT → CTT	0	61
10	66	f	III	6	190	CCT → CTT	20	12
11	19	m	IV	8–9	n.d.	n.d.	36	16
12	58	f	IV	6	210	AAC → CAC	0	6
13	44	m	IV	6	202	CGT → CTT	17	12
14	65	f	IV	5	151	CCC → CTC	56	12
Mixed gliomas								
15	28	m	II	7				
16	33	m	III	8–9				
17	49	m	III	5				
18	35	m	III	6				

f, female; m, male; nr, no recurrence; n.d., sequence not determined due to technical problems; * Interval, interval between first tumour operation and tumour re-operation in months.

tumours including breast and lung carcinomas (for references, see *TP53* database by Hollstein and coworkers [12]). Whether this over-representation in exon 6 indicates that this is a typical location for *TP53* mutations in oligodendrogliomas or merely an accidental finding cannot be determined from the present investigation, and more data are needed. Codons 151 and 176 in exon 6 have been reported to be mutated in astrocytomas [13, 14] as have mutations

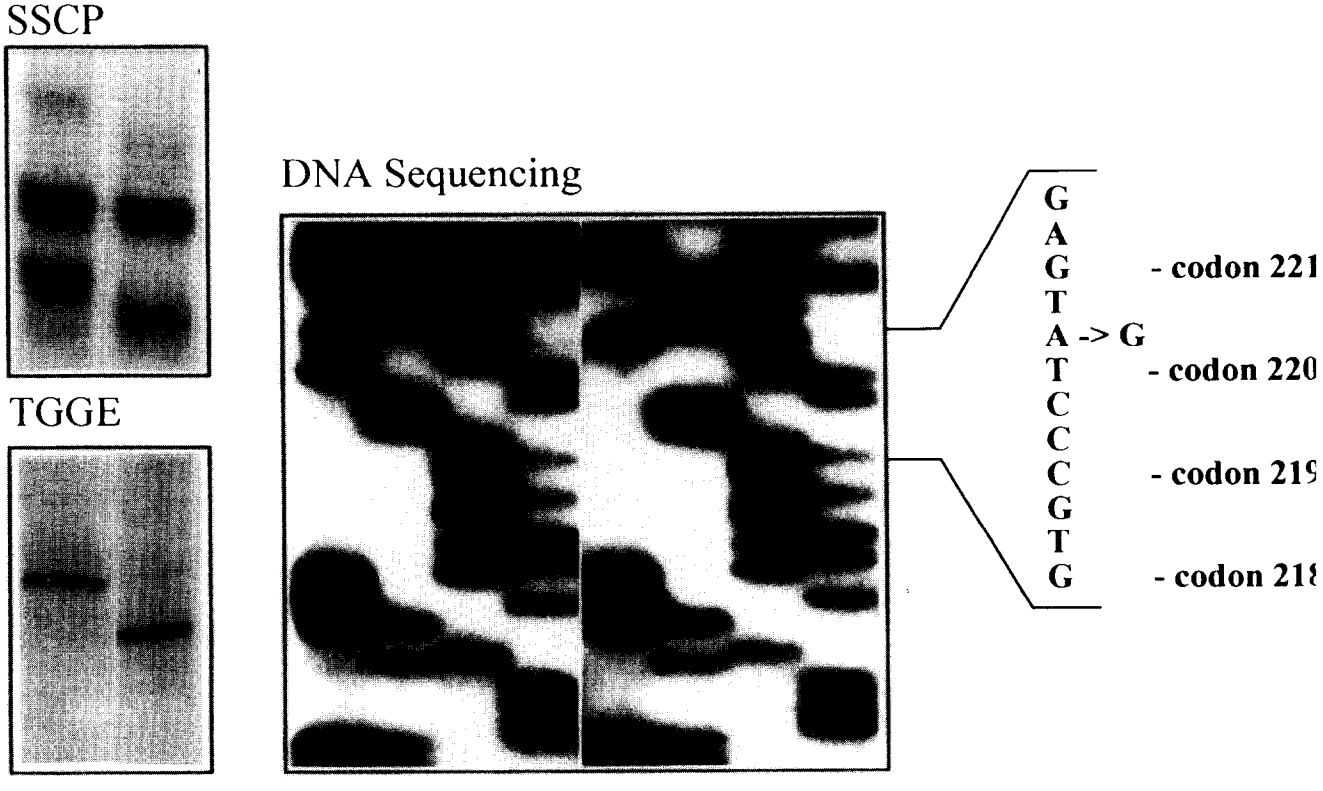

Figure 4. Example of a mutation in exon 6. p53 wild type on left lanes, mutations right. Note the loss of heterozygosity. Recurrence of case 105–90.

in codon 239 of exon 7 [15]. In accordance with mutations reported previously in oligodendrogliomas, none of the mutations affected the mutational hot spot at codon 248 [10].

Although p53 protein accumulation and *TP53* mutations were significantly correlated in the present study ($P < 0.001$, Table 1), a discordance between the two variables was found in 23 cases, with detection of a *TP53* mutation without p53 protein accumulation in 7 cases and detection of protein without muation in 16 cases.

The discrepancy of p53 protein accumulation without the presence of mutations was previously found in various studies on astrocytomas [5, 16–18]. The number of cases with p53 protein accumulation exceeded that of mutations by a factor of 1.3 [18] to 2 [17]. Several reasons may be responsible for this effect. Firstly, the mutation may be located outside the investigated regions (in most studies exons 5–8, covering codons 126–306). A re-investigation of 560 mutations revealed that only 87% of the mutations were located in exons 5–8, whereas exon 4 was affected in 8% and exon 10 in 4% [19]. Secondly, there is evidence that wild type p53 conformation is sensitive to growth conditions and cell type; a shift to a mutant-like conformation has been observed in growth stimulated lymphocytes and marrow blast cell populations [20]. Thirdly, the *MDM-2* gene, which was shown to be amplified in human sarcomas [21], might play a role in the accumulation of wild type p53 protein. The gene product of *MDM-2* forms tight complexes with the p53 protein, thereby inactivating the normal growth regulatory functions of p53 protein. In the present study, *MDM-2* was not investigated, but in a study of 17 glioblastoma multiforme no correlation between re-arrangement or amplification of the *MDM-2* locus was found, including cases with p53 protein accumulation, but lacking *TP53* gene mutations [2]. Thus, *MDM-2* gene amplification might not play a significant role in gliomas.

Concerning the discrepancy between cases with *TP53* mutation but without p53 protein accumulation, deletions or insertions of bases may result in non-sense mutations and consequently to truncated p53 protein, which may not be detected immunohistochemically. In the present study, one case without p53 accumulation (case 3) was found to harbour a deletion in codon 201 leading to a frameshift. In the other 6 cases no obvious reason for the negative immunoreactivity was found. Koga and coworkers [16], who also found a lack in immunoreactivity in one of the cases, suspected protein instability, assuming that the lack of immunoreactivity was not due to technical reasons. Another possible explanation for the negative immunoreactivity could be the presence of additional non-sense mutations outside the investigated region. Further, depending on the location of the mutation and the amino acid change, some mutations might not affect the proteolysis of the p53 protein and thus would not lead to increased levels of p53 protein. One of the seven mutations without p53 protein accumulation was located in exon 7 and has been previously reported in an anaplastic astrocytoma [15]. Unfortunately, p53 protein was not investigated in this previous study.

In conclusion, *TP53* gene mutations appear to be a late event in the tumorigenesis by oligodendroglomas, with mutation frequencies comparable with those reported for astrocytomas only demonstrated in anaplastic oligodendrogliomas. Since p53 protein accumulation but not *TP53* gene mutations, were found to correlate with tumour grade, p53 protein accumulation may have some diagnostic relevance in oligodendrogliomas.

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