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RESEARCH ARTICLE

High Frequency of *TP53* Mutations in Juvenile Pilocytic Astrocytomas Indicates Role of *TP53* in the Development of These Tumors

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In adults, the *TP53* tumor suppressor gene is frequently mutated in astrocytic brain tumors which is supposed to represent an early event in their development. In juvenile pilocytic and low-grade astrocytomas, however, *TP53* mutations have until now been reported as rare, which has led to the suggestion that these tumors may follow a different molecular pathogenesis with an involvement of genes other than *TP53*. Our analysis of 20 pilocytic and two low-grade astrocytomas of childhood, based on a comprehensive denaturing gradient gel electrophoresis (DGGE) mutation detection assay of the entire coding region, including all splice site junctions of *TP53*, showed mutations considered as causative in 7 of the 20 (35%) pilocytic astrocytomas and in one of the two low-grade astrocytomas. Our finding is significantly different from the mutation frequency of 1.3% (2/155) previously reported for these tumor types. This may be attributed to the mutation detection system used, which also detects mutations occurring outside the evolutionary conserved region of *TP53*. Our results suggest that, contrary to the present notion, *TP53* mutations may well play a role in the development of juvenile astrocytomas. Furthermore, no mutations were found in tumors of patients with progression of residual tumor after postoperative follow-up. This suggests that *TP53* mutations may be associated with less aggressive forms of juvenile astrocytomas, analogous to the situation in adult astrocytomas.

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Introduction

The *TP53* tumor suppressor gene is the most commonly mutated gene in human neoplasms (8, 18), including tumors of the brain (reviewed in 1, 15). The gene, located at band p13.1 of chromosome 17, consists of 11 exons of which exon 1 is noncoding (11). *TP53* codes for the 393 amino acid residues-long p53 protein that plays an essential role in DNA damage-induced cell cycle arrest and apoptosis. Most of the evolutionarily conserved region of *TP53* lies within exons 5 to 8, containing the DNA-binding domain of p53 (8, 13, 18, 25). Studies to date, have therefore generally been restricted to the analysis of this region.

Astrocytic tumors are the most common primary brain tumors in children, accounting for 30-40% of all neoplasms of the central nervous system (4). In contrast to astrocytic tumors in adults, those in childhood are most frequently of the pilocytic type (5). Pilocytic astrocytomas constitute a well-defined histopathological entity, differing from the low-grade astrocytomas (WHO grade II astrocytoma), that occur less frequently in children. Both juvenile pilocytic and low-grade astrocytomas tend to be of a slowly growing, clinically more benign nature (4). Previous studies have indicated that *TP53* mutations represent an early event in the genesis of astrocytomas in adults (reviewed in 15). These adult astrocytomas may be further divided into primary and secondary glioblastomas. *TP53* mutations are rare in the aggressive, more rapidly developing primary (de novo) glioblastomas, but do occur in the early stages of secondary glioma progression. This suggests, that there are two different genetic pathways involved in the evolution of glioblastomas (27). According to the literature, as summarised in Table 1, mutation of *TP53* seems to be a rare event in pilocytic and low-grade astrocytomas of childhood. It has therefore been suggested, that the molecular pathogenesis of these tumor types is different from that of most astrocytic brain tumors in adults and would involve genes other than *TP53* (26).

Study	Method applied[1]	TP53 exons	Astrocytomas (n = 155)[2]	Mutation[3]
Mashiyama et al.,1991 (17)	SSCP	2-11	6(lga)	none
Hunter et al.,1993 (10)	SSCP	5,7,8	8(pa)	none
Ohgaki et al.,1993 (19)	SSCP	5-8	12(pa)	none
Lang et al.,1994 (12)	SSCP	2-11	7(pa)	exon 4: Pro47-Ser
Litofsky et al.,1994 (14)	SSCP	5-8	17(pa)	none
			4(lga)	none
Rasheed et al.,1994	SSCP	5-8	28(lga)	exon 6: Arg213-Arg
Felix et al.,1995 (2)	SSCP	4-8	17(lga)	none
Schiffer et al.,1995 (23)	SSCP	5-8	11(pa)	none
Willert et al.,1995 (28)	DGGE	4-9	20(pa)	exon 7: Trp248-Arg*
Patt et al.,1996 (20)	SSCP	5-8	7(pa)	exon 9: Asp324- Asp
			18(lga)	exon5: Pro151-Leu*

[1] Abbreviations of mutation detection methods used are as follows: SSCP = single-stranded conformation polymorphism, DGGE = denaturing gradient gel electrophoresis.
[2] Juvenile astrocytomas are classified into the following two categories; pa = pilocytic astrocytomas (n = 82), lga = low-grade astrocytomas (n = 73).
[3] Mutations of a possibly causative nature are represented with a (*), the remaining 3 mutations are non-causative (2 silent and 1 rare polymorphism).

Table 1. Literature summary of *TP53* mutation screening of juvenile pilocytic and low-grade astrocytomas,ordered according to the year of study.

Using an assay based on denaturing gradient gel electrophoresis (DGGE), we have analyzed a series of 20 paraffin-embedded pilocytic astrocytomas and two low-grade astrocytomas of childhood for mutations in the entire coding region, including all splice site junctions, of the *TP53* gene. Here we present the results of this study.

Materials and Methods

Tumor samples. Formalin-fixed paraffin-embedded tumor material was obtained from 22 patients, all Dutch Caucasians with the exception of one patient from African ethnic origin, who underwent surgery between 1975 and 1995 at the University Hospital, Groningen, The Netherlands. At the time of primary surgery, all patients were in the pediatric age group (0-16 years). The tumors were histologically classified according to WHO criteria and included 20 pilocytic astrocytomas and two low-grade (fibrillary) astrocytomas. The 20 pilocytic astrocytomas were further classified after post-operative follow-up, done by MRI- or CT-scanning. For 9 patients surgery resulted in total tumor resection. For 7 patients with residual tumor, follow-up scans revealed that the tumor showed no progression and remained stable. For 4 patients progression of the tumor was found after 12 months of the primary surgery. The clinical follow-up period of the patients ranged from 12 to 133 months.

Genetic Analysis. DNA was extracted from 5 x 10 μm sections of paraffin-embedded tumor by the chelex-boiling method (24). The entire coding region, including all splice site junctions, of the *TP53* gene were amplified in 12 amplicons using nested PCR primers and conditions described elsewhere (6). All amplicons were electrophoresed in a 9% polyacrylamide gel containing 5% glycerol and a 35-75% urea-formamide (UF) denaturing gradient (100% UF = 7M Urea/40% deionised formamide). The gels were stained with ethidium bromide and photographed under an UV transilluminator. Samples showing mobility shifts in the DGGE gels, were subjected to single-stranded automated sequencing on an ABI Prism 377 DNA sequencer, using a non-GC-clamped primer and the Perkin Elmer dye terminator sequencing kit. All mutations were confirmed either by sequencing from the reverse primer or by restriction digestion. PCR artifacts were excluded by independent PCR-DGGE of mutant amplicons.

Results

In our study, 17 *TP53*-DGGE variations were found in 13 of the 22 astrocytomas. The DGGE-patterns are depicted in Figure 1. Direct sequencing revealed DNA sequence variations, listed in Table 2 according to the exon/ intron in which they occur. Twelve of the 17 variations occurred outside exons 5 to 8. Eight mutations (7 in pilocytic astrocytomas and 1 in a low-grade astrocytoma) are considered as being causative, as they result either directly in a truncated protein (2 nonsense mutations) or in a non-conservative amino acid substitution (6 missense mutations). A non-conservative amino acid substitution is defined as a replacement of an amino acid by another with different chemical properties. The six possibly causative missense mutations are currently under further investigation by computational protein

Tumor	Tumor Type[1]	Mutation	Exon/Intron[2]	Codon	Amino Acid Change	Mutation Type[3]
B13	pa(r)	CAG-TAG	2	5	Gln-Stop	cnm
B11	pa(r)	CCG-TCG	4	47	Pro-Ser	rp
B6	pa(s)	CCA-TCA	4	67	Pro-Ser	cmm
B21	pa(s)	ACA-ATA	4	81	Thr-Ile	cmm
B21	pa(s)	GCC-GTC	4	84	Ala-Val	mm
B28	pa(s)	GCC-GTC	4	84	Ala-Val	mm
B26	pa(r)	TGG-TAG	4	91	Trp-Stop	cnm
B23	pa(s)	AAG-ATG	4	120	Lys-Met	cmm
B12	lga	ACT-ATT	4	123	Thr-Ile	cmm
B1	pa(r)	TAC-TAT	5	126	Tyr-Tyr	silent
B8	pa(r)	AAG-GAG	5	132	Lys-Glu	cmm
B7	pa(r)	deletion gg	int5	+13,14	-	intd
B1	pa(r)	GTG-GGG	6	218	Val-Gly	mm
B24	pa(s)	c-t	int8	+6bp	-	intbc
B1	pa(r)	AAG-AAA	9	320	Lys-Lys	silent
B25	pa(s)	GGG-GAG	10	356	Gly-Glu	cmm
B1	pa(r)	CGC-CAC	11	379	Arg-His	mm

[1]Tumor type abbreviations are as follows; pa = pilocytic astrocytoma, lga = low grade astrocytoma. Postoperative follow-up classifications in closed brackets are as follows; r = totally resected, s = stable residual tumor.
[2] An intronic mutation is indicated by the abbreviation int.
[3] Mutation types are abbreviated as follows; cnm = causative nonsense mutation, cmm = possibly causative missense mutation, mm = possibly non-causative missense mutation, rp = previously reported rare polymorphism, silent = silent mutation, intd = possibly non-causative intronic deletion, intbc = possibly non-causative intronic base change. The terms possibly causative and possibly non-causative are defined in the text.

Table 2. *TP53* mutations found in 20 pilocytic and 2 low-grade juvenile astrocytomas.

analysis to determine the nature of these mutations.

Nine possibly non-causative mutations were found to occur in six of the pilocytic astrocytomas, including two silent mutations, one previously described rare polymorphism, four conservative amino acid changes occurring outside the p53 DNA-binding domain and two intronic mutations not involved in the splicing mechanism as defined by the NetGene2 prediction program (7). Tumor B1 presented with four such mutations, 2 silent (codons 126 and 320) and 2 conservative amino acid substitutions (codons 218 and 379). The common exon 4 polymorphism Arg72-Pro, was found as an arginine homozygote or hemizygote in 10 of the 22 astrocytomas, as an arginine/ proline heterozygote in 8 tumors, and as a proline homozygote or hemizygote in 4 tumors.

No mutations (causative or non-causative) were found to occur in the four pilocytic astrocytomas showing progression of residual tumor. Six of the seven pilocytic astrocytomas with residual tumor that remained stable after postoperative follow-up, however, had one or more *TP53* mutations, four with possibly causative mutations (B6, B21, B23 and B25) and two with possibly non-causative mutations (B24 and B28).

Discussion

By using a comprehensive DGGE mutation detection assay to screen the entire *TP53* gene coding region, including all splice site junctions, in 20 juvenile pilocytic and two low-grade astrocytomas, we found that seven of the 20 pilocytic astrocytomas (35%) and one of the two low-grade astrocytomas had a causative *TP53* mutation. The frequency of *TP53* mutations in our study is significantly higher than previously reported in studies of these tumor types. A compilation of data from the literature (Table 1) gives a mutation frequency of 1.3% (2/155). Based on that, it has been proposed that mutation of *TP53* is a rare event in the genesis of these tumors (14, 26). In almost all of the previous studies, however, single-stranded conformation polymorphism (SSCP) analysis has been used for mutation detection. The sensitivity of that method is significantly lower (22) than that of the DGGE-method which we applied. Moreover, most of the previous studies were restricted to the analysis of the evolutionarily conserved region of *TP53* (exons 5-8). The high mutation frequency we found, may thus be attributed to our highly sensitive and comprehensive mutation detection system, which detected a majority of mutations (12/17 or 71%) outside the evolutionarily conserved region. Although numbers are small, our results suggest that, contrary to the present notion, *TP53* mutations may well play a role in the development of childhood astrocytomas.

The homozygous (or hemizygous) missense mutation Pro47-Ser, occurring in our only patient with an African ethnical background (B11), was previously reported as the first possibly causative mutation found in a pilocytic astrocytoma (12). Functional studies, however, proved this mutation to be a rare germline polymor-

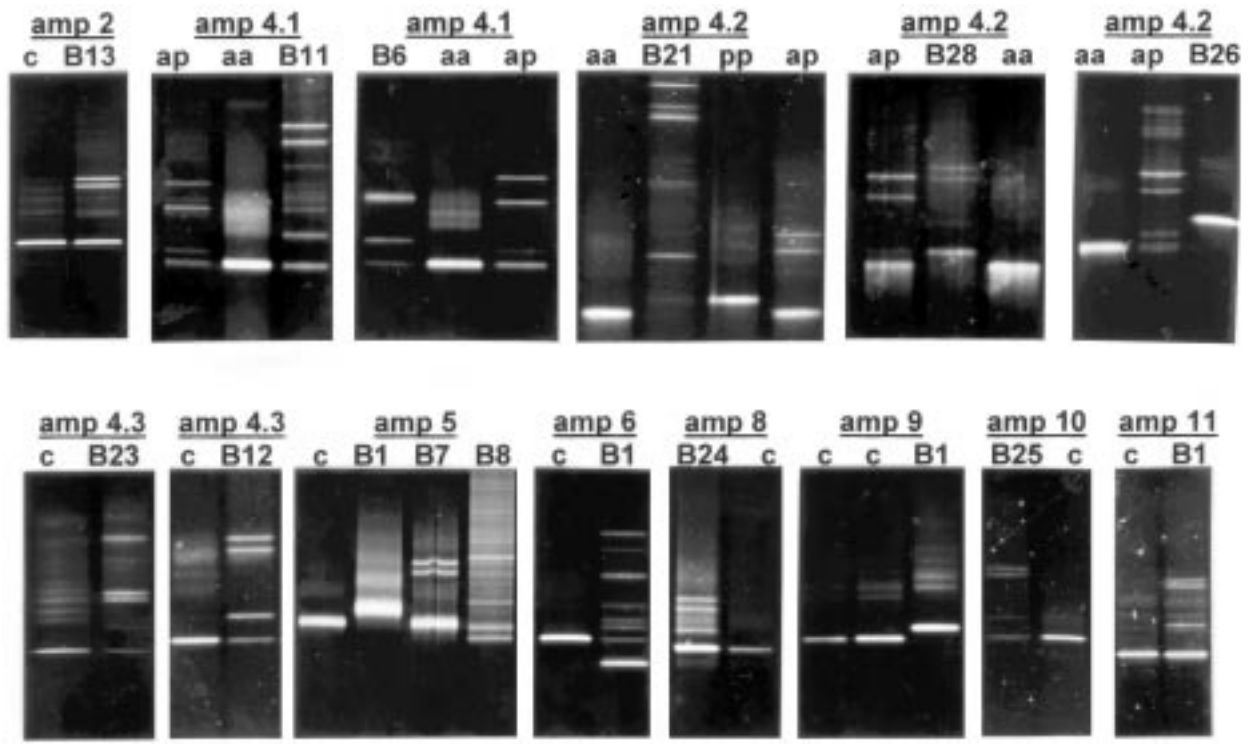

Figure 1. Aberrant DGGE banding patterns for all *TP53* mutations found in 20 pilocytic and two low-grade juvenile astrocytomas (Table 2), listed according to the amplicon (amp) in which they were found. Abbreviations used are as follows; c, control, and B, relevant brain tumor. The common codon 72 polymorphism (16) occurs within the overlapping region of amplicons 4.1 and 4.2, and is represented by the following abbreviations; aa, ArgArg; ap, ArgPro; and pp, ProPro. The mutations occurring in samples B26 (amplicon 4.2), B1 (amplicon 5) and B1 (amplicon 9) appear as a single aberrant DGGE band and are therefore hemizygous or homozygous mutations. All remaining mutations appear as four bands (two homoduplex and two heteroduplex) as seen in samples B12 (amplicon 4.3) and B8 (amplicon 5), and are heterozygous. DGGE analysis was performed on DNA extracted from paraffin-embedded material in which the proportions of normal and mutant cells per sample may vary. This may explain the differences in intensities of the homozygous normal and mutant bands in samples B24 (amplicon 8) and B1 (amplicon 11). An excess of normal DNA will result in the mutant alleles being exhausted during heteroduplex formation and therefore loss of the homoduplex mutant band. This may be the explanation for the patterns from B13 (amplicon 2), B7 (amplicon 5) and B25 (amplicon 10). Presence of more mutant than normal cells will result in loss or weaker intensity of the homozygous normal band. B28 (amplicon 4.2), B23 (amplicon 4.3) and B1 (amplicon 6) may represent this situation. In B6 (amplicon 4.1), the heteroduplex bands have the same melting profile and therefore appear as a single DGGE band. The large number of bands seen in samples B11 (amplicon 4.1) and B21 (amplicon 4.2) results from the presence of two variations per amplicon.

phism with an allele frequency of 4.7% among African-Americans (3). The nonsense mutation occurring in the pilocytic astrocytoma B13 in exon 2 (Gln5-stop) and resulting in a severely truncated protein of only four amino acid residues, is the most 5′ situated *TP53* nonsense mutation reported to date (*TP53* database, 9). A clustering of four non-causative mutations in a single tumor, as was found in tumor B1, may be the result of "stuttering" during DNA synthesis, followed by defective mismatch repair.

No mutations were found in the residual tumors showing progression of the tumor during postoperative follow-up. The time to progression ranged from 2 to 12 months. Of the 7 stable residual tumors, 4 (57%), however, presented with a possibly causative *TP53* mutation. The clinical follow-up period of the patients with tumors with a mutation ranged from 32 to 81 months (average 59 months). Although random variation cannot be excluded as a possible cause, this observation might suggest an association of *TP53* mutations with the development of less aggressive forms of this tumor type. If so, there is in this respect some parallel to the development of adult astrocytomas (27). In the stable, less aggressive, juvenile pilocytic astrocytomas, mutation of *TP53* seems to be an early event, as also observed in adult secondary glioma formation. However, in the pro-

gressive juvenile pilocytic astrocytomas, as in adult aggressive primary glioblastomas, mutation of *TP53* is a rare event.

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References

1. Bogler O, Huang HJS, Kleihues P, Cavenee W (1995) The p53 gene and its role in human brain tumours. *GLIA* 15: 308-327
2. Felix CA, Slavc I, Dunn M, Strauss EA, Phillips PC, Rorke LB, Sutton L, Bunin GR, Biegel JA (1995) p53 gene mutations in pediatric brain tumours. *Med Pediat Oncol* 25: 431-436
3. Felley-Bosco E, Weston A, Cawley HM, Bennett WP, Harris CC (1993) Functional studies of a germ-line polymorphism at codon 47 within the p53 gene. *Am J Hum Genet* 53: 752-759
4. Giannini C, Scheithauer BW (1997) Classification and grading of low-grade astrocytic tumors in children. *Brain Pathol* 7: 785-798
5. Gilles FH, Leviton A, Hedley-White ET, Sobel E, Tavare CJ, Sobel RS, Rorke LB (1992) Childhood brain tumors that occupy more than one compartment at presentation. *J Neurooncol* 14: 45-56
6. Hayes VM, Bleeker W, Verlind E, Timmer T, Karrenbeld A, Plukker JT, Marx MP, Hofstra RMW, Buys CHCM. A comprehensive TP53-denaturing gradient gel electrophoresis assay also applicable to archival paraffin-embedded tissue. *Diagn Mol Pathol*, in press
7. Hebsgaard SM, Korning PG, Tolstrup N, Engelbrecht J, Rouze P, Brunak S (1996) Splice site prediction in Arabidopsis thaliana DNA by combining local and global sequence information. *Nucleic Acids Res* 24: 3439-3452
8. Hollstein M, Sidransky D, Vogelstein B, Harris CC (1991) p53 mutations in human cancers. *Science* 253: 49-53
9. Hollstein M, Shomer B, Greenblatt M, Soussi T, Hovig E, Montesano R, Harris CC (1996) Somatic point mutations in the p53 gene of human tumors and cell lines: updated compilation. *Nucl Acids Res* 24: 141-146
10. Hunter SB, Bandea C, Swan D, Abbott K, Varma A. (1993) Mutations in the p53 gene in human astrocytomas: Detection by single-stranded conformation polymorphism analysis and direct DNA sequencing. *Mod Pathol* 6: 442-445
11. Lamb P, Crawford LV (1986) Characterization of the human p53 gene. *Mol Cell Biol* 6: 1379-1385
12. Lang FF, Miller DC, Pisharody S, Koslow M, Newcomb EW (1994) High frequency of p53 protein accumulation without p53 gene mutation in human juvenile pilocytic, low grade and anaplastic astrocytomas. *Oncogene* 9: 949-954
13. Levine AJ, Momand J, Finlay CA (1991) The p53 tumour supressor gene. *Nature* 351: 453-456
14. Litofsky NS, Hinton D, Raffel C (1994) The lack of a role for p53 in astrocytomas in pediatric patients. *Neurosurgery* 34: 967-973
15. Louis DN (1994) The p53 gene and protein in human brain tumors. *J Neuropathol Exp Neurol* 53: 11-21
16. Matlashewski GJ, Tuck S, Pim D, Lamb P, Schneider J, Crawford LV (1987) Primary structure polymorphism at amino acid residue 72 of human p53. *Mol Cell Biol* 7: 961-963
17. Mashiyama S, Murakami Y, Yoshimoto T, Sekiya T, Hayashi K (1991) Detection of p53 gene mutations in human brain tumors by single-strand conformation polymorphism analysis of polymerase chain reaction products. *Oncogene* 6: 1313-1318
18. Nigro JM, Baker SJ, Preisinger AC, Jessup JM, Hostetter R, Cleary K, Bigner SH, Davidson N, Baylin S, Devilee P, Glover T, Collins FS, Weston A, Modali R, Harris CC, Vogelstein B (1989) Mutations in the p53 gene occur in diverse human tumor types. *Nature* 342: 705-708
19. Ohgaki H, Eibl RH, Schwab M, Reichel MB, Mariani L, Gehring M, Petersen I, Hoell T, Wiestler OD, Kleihues P (1993) Mutations of the p53 suppresser gene in neoplasms of the human nervous system. *Mol Carcinogen* 8: 74-80
20. Patt S, Gries H, Giraldo M, Cervos-Navarro J, Martin H, Janisch W, Brockmoller J (1996) p53 gene mutations in human astrocytic brain tumors including pilocytic astrocytomas. *Hum Pathol* 27: 586-589
21. Rasheed BKA, McLendon RE, Herndon JE, Friedman HS, Friedman AH, Bigner DD, Bigner SH. Alterations of the TP53 gene in human gliomas (1994) *Cancer Res* 54: 1324-1330
22. Scheffield VC, Beck JS, Kwitek AE, Sandstrom DW, Stone EM (1993) The sensitivity of single-strand conformation polymorphism analysis for the detection of single base substitutions (1993) *Genomics* 16: 325-332
23. Schiffer D, Cavalla P, Di Sapio A, Giordana MT, Mauro A (1995) Mutations and immunohistochemistry of p53 and proliferation markers in astrocytic tumors of childhood. *Child's Nerv Syst* 11: 517-522
24. Stein A, Raoult D (1992) A simple method for amplification of DNA from paraffin-embedded tissue. *Nucleic Acids Res* 20: 5237-5238
25. Vogelstein B, Kinzler KW (1992) p53 finction and dysfunction. *Cell* 70: 523-526
26. von Deimling A, Louis DN, Menon AG, von Ammon K, Peterson I, Ellison D, Wiestler OD, Seizinger BR (1993) Deletions on the long arm of chromosome 17 in pilocytic astrocytoma. *Acta Neuropathol* 86: 81-85
27. Watanabe K, Tachibana O, Sato K, Yonekawa Y, Kleihues P, Ohgaki H (1996) Overexpression of the EGF receptor and p53 mutations are mutually exclusive in the evolution of primary and secondary glioblastomas. *Brain Pathol* 6: 217-224