Recurrent astrocytoma in a child: A report of cytogenetics and *TP53* gene mutation screening¹

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An 8-year-old girl presented with a cerebral tumor and 3 recurrences within 15 months. The primary tumor was a low-grade astrocytoma, but the recurrences showed progressively malignant phenotypes with increasing mitotic activity and MIB-1 labeling indices. Radiotherapy was given between the first and the second recurrences. Cytogenetic analysis of the first and the second recurrences showed abnormal karyotypes. There seemed to be 2 common breakpoints in these 2 recurrences. *TP53* gene mutation screening, using comprehensive denaturing gradient gel electrophoresis, revealed among others a possibly causative mutation of exon 5 in 3 of 4 tumor samples. The meaning of *TP53* mutations in low-grade astrocytomas is still unclear, but the highly abnormal karyotypes, which are unusual in these tumors, probably provide genetic evidence for the unexpected aggressive behavior of the tumor in this patient. *Neuro-Oncology* 2, 184-189, 2000 (Posted to *Neuro-Oncology* [serial online], Doc. 00-013, May 16, 2000. URL <neuro-oncology.mc.duke.edu>)

The most common histologic type of astrocytic tumor in children is the pilocytic astrocytoma, but diffuse astrocytomas of all grades of malignancy are observed as well (Giannini and Scheithauer, 1997). They have a predilection for the cerebellum and brainstem, but may occur elsewhere in the CNS (Cohen and Duffner, 1994). Outcome after surgery is in most cases rather

favorable (Lapras et al., 1986; Sgouros et al., 1995). Cystic astrocytomas often can be resected totally and seldom recur. Depending on the grade and classification of the tumor, radiation therapy may be added (Lapras et al., 1986). Sometimes the biologic behavior of these astrocytic tumors is rather unpredictable, and follow-up of these patients is always necessary. In the case of tumor recurrence, a second-look surgical intervention is needed.

The current study describes the histology, including MIB-1 labeling, cytogenetic findings, and the results of *TP53* mutation analysis, in a patient with a peculiar clinical course and unusual histology.

Clinical History and Histology

The patient, an 8-year-old girl, was born without difficulty after an uncomplicated pregnancy. Motor milestones and mental development were normal until the age of 5. From then on, she developed an impulsive and unpredictable behavior, and at age 6 she had school problems. She started to complain about headache, nausea, and vomiting when she was 8 years old. The clinical history was completed with complaints of paresthesia in her left arm, painful eyes, and headtilting. Neurologic examination revealed a torticollis to the right, vision loss, and papilledema. MRI of the brain showed a multilobular cystic mass in the left frontal lobe with edema, a shift to the right of the medial structures, compression of the third ventricle, and a slight hydrocephalus. A subtotal resection of the tumor mass was done. Histologically, the specimen showed 2 different components. One consisted of microcystic tumor tissue with low cellularity, compatible with low-grade astrocytoma (Fig. 1A). The second component consisted of a more cellular, solid proliferation of medium-sized round cells with little pleomorphism (Fig. 1B). There was infiltration in the overlying cortex and meninges. There was virtually no mitotic activity. The MIB LI³ was 0.17.

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³Abbreviations used are as follows: DI, DNA index; FISH, fluorescence in situ hybridization; LI, labeling index.

Fig. 1. Histology of the primary tumor showing areas with low (A) and higher (B) cellularity; second recurrence, after radiotherapy (C), and third recurrence showing undifferentiated tumor tissue (D). Hematoxylin and eosin. A, B, and C, original magnification X200; D, original magnification X400.

Necrosis was focally present, primarily in the area with low cellularity. A diagnosis of low-grade astrocytoma with focally increased cellularity was made.

After surgery, the patient developed cognitive problems, mostly involving verbal expression and dyslexia, but without distinct aphasia. She suffered from anxiety problems and complained about paresthesias in the left arm. Follow-up MRI showed the resection cavity with enhancement of gadolinium at the edges, but without evidence for tumor recurrence (stable disease). Six months postoperatively, complaints of vomiting and headache recurred, and clinical examination revealed papilledema again. MRI showed a mass in the subdural space and intraparenchymally in the left frontal lobe. A second-look craniotomy was done, and a hemorrhagic tumor mass was resected. Histologic examination showed areas similar to the initial tumor (coded Rec. 1a). In addition, areas with high cellularity and nuclear atypia were observed (coded Rec. 1b), with mitotic activity up to 8/2 mm², whereas the MIB LI had increased to 2.35, both indicating progression in malignancy. Radiation therapy was started: 59 Gy in 33 fractions, shrinking field. Within 4 months the complaints recurred, and MRI showed an increasing volume of the frontally localized cerebral cyst and enhancement of gadolinium in its wall. Aspiration of cyst fluid showed a

very high protein count of 46 g/liter. An Ommaya reservoir was placed, and repeated aspirations were done to diminish the volume of the cyst. One year after the initial presentation, the cyst-plus-tumor mass was resected again. This second recurrence (coded Rec. 2), after radiation therapy, showed more pleomorphism and multinucleation of tumor cells, but no mitotic activity (Fig. 1C). The MIB LI was 0.21, in the range of the first tumor sample.

Despite the resection, the patient developed a progressive frontal syndrome. A CT scan showed an enlarging cyst with increased enhancement and progression of tumor mass, compression of the foramen of Monro, and obstructive hydrocephalus. A fourth surgical exploration was done with resection of a well vascularized tumor mass (coded Rec. 3) out of the cyst wall, and a ventriculo-peritoneal drain was placed. The histology, 15 months after the initial diagnosis, showed densely cellular, undifferentiated tumor tissue (Fig. 1D) with brisk mitotic activity (up to 23/2 mm²), a MIB LI of 8.59, and extensive infiltration in the meninges. Within a month, identical complaints of headache and behavioral disturbances reappeared. No adequate curative therapy could be given any more. The patient died 4 months later. Permission for autopsy was refused.

Materials and Methods

All tumor samples were stained for MIB-1 (see below). Samples of the first (Rec. 1a and 1b together) and the second recurrence (Rec. 2) could be processed for tissue culture, cytogenetics, FISH, and DNA flow cytometry. In addition, all tumor samples except the third recurrence could be screened for mutations in the *TP53* gene.

MIB-1 Labeling

MIB-1 labeling was performed on formalin-fixed paraffin-embedded tissue of all the resection material. Each section measuring 5 µm was cut, floated in a water bath of 50°C, and mounted on aminopropylethoxysilane (APES)-coated slides, which were placed at 60°C for 15 min and dried overnight in an oven at 37°C. Wet autoclaving was used twice for antigen retrieval. The slides were then incubated with primary MIB-1 antibody (Immunotech, Villeponte, France) diluted 1:400. Two-step immunostaining was performed using antimouse biotin and alkaline phosphatase-conjugated streptavidin, according to the manufacturer's procedure of the Biogenex kit (Biogenex, San Ramon, CA). NBT/BCIP (4-nitroblue tetrazolium chloride and bromo-chloro-indolyl-phosphate; Boehringer Mannheim, Mannheim, Germany) was used as substrate. Ten high-power (×400) microscopic fields were randomly selected from each tumor slide, and the cells were counted. MIB-1 LI was calculated as the number of positive cells divided by the total number of cells.

Cytogenetics

For cytogenetic analysis, part of the tissue was minced with scalpels, incubated in a collagenase-deoxyribonuclease solution, and cultured in RPMI 1640 supplemented with

Table 1. Karyotype description of the first and the second recurrence

First recurrence (Rec. 1a + b together):

43~45,XX,-18[cp3]/40,XX,add(1)(q21),del(4)(q21q31),-5,-13,-14,der(14)t(?9;14).ish add(14)(p13)(wcp9-,wcp14+),-15,-17,-18,-21,-22,+2mar[1]/79~80,idemx2,-del(4)(q21q31),+14,-der(14)t(?9;14).ish add(14)(p13)(wcp9-,wcp14+),+21,-2mar[cp2]/46,XX[19].

Second recurrence (Rec. 2):

44~46,X,del(X)(q23),del(2)(p13),add(3)(q29),add(6)(q21),der(14)t(?9;14).ish t(14;14)(p13;q23)(wcp9-,wcp14+,D14S308++),add(15)(q24),t(17;18)(q11;p11.3),dd(17)(q22),add(22)(q13)[cp7]/43~46,XX,t(1;7)(q21;p22)del(7)(q32),t(5;8)(p15;q13),add(8)(q13),t(11;16)(q13;p12),inv(12)(q13q15),del(16)(q21)[cp2]/46,XX,del(6)(p22)[cp2].

16% fetal calf serum, glutamine, and antibiotics. After short-term culturing, the cells were harvested, and chromosome preparations were made according to standard cytogenetic techniques. The chromosomes were G-banded using trypsin/pancreatin, and karyotypes were described according to ISCN (Standing Committee on Human Cytogenetic Nomenclature, 1995).

DNA Flow Cytometry

For DNA flow cytometry, we used a method developed by Vindelov et al. (1983). Nuclei were isolated using a detergent-trypsin method, exposed to ribonuclease, and incubated with propidium iodide. The intensity of the fluorescent signal was measured using a Coulter Epics Elite flow cytometer (Coulter, Hialeah, FL). For each sample, 30,000 events were analyzed.

FISH

For FISH studies, specific libraries for chromosomes 9 and 14 (commercially available from Cambio, Downer's Grove, IL) and the Tel 14q DNA probe (P5428-DIG; ONCOR, Gaithersburg, MD) were used. In situ hybridization was carried out essentially according to the manufacturer's protocol.

TP53 Gene Analysis

For genetic analysis of the *TP53* gene, DNA was extracted from $5 \times 10 \mu\text{m}$ sections of paraffin-embedded tumor tissue using the chelex boiling method (Stein et al., 1992). The entire coding region, including all splice site junctions, of the *TP53* gene was amplified in 12 amplicons using nested polymerase chain reaction primers under conditions previously described (Hayes et al., 1999a, 1999b). All amplicons were electrophoresed in a 9% polyacrylamide gel containing 5% glycerol and a 35% to 75% UF denaturing gradient (100% UF = 7 M urea/40% deionised formamide). Electrophoretic conditions used were as described elsewhere (Hayes et al., 1999a, 1999b).

Results

Results of the cytogenetic analysis are described in Table 1. Figs. 2 and 3 show karyotypes of Rec. 1a and b, and Rec. 2, respectively. In Rec. 1a and b, only one aberrant cell, 40,XX, could be karyotyped. However, 2 more cells 79~80,idemx2, were found, indicating that most of the aberrations in the first cell were clonal. In addition, Rec. 2 showed many nonclonal numerical and structural aber-

Fig. 2. Representative karyotype of the first recurrence.

Fig. 3. Representative karyotype of the second recurrence.

rations, typically chromosomal rearrangements of the balanced type.

Despite the radiation therapy, 2 breakpoints seemed to be common to both karyotyped recurrences: 1q21 and 14p13. Derivative chromosomes revealing the 1q21 breakpoints were not identical, but the structural rearrangement involving 14p13 seemed similar in both cases. FISH analysis revealed the presence of a t(14;14) in Rec. 2. However, this was not detected in Rec. 1a and b.

The DNA flow cytometry histograms of both Rec. 1a and 1b revealed a hypertriploid peak (DI 1.75 and 1.78), which is in agreement with the clone of 79~80 chromosomes. Cells with 40 and 43~45 chromosomes were present in low numbers and were in the diploid range, probably explaining why they were not detected in the DNA histogram as a peak separate from the normal diploid peak. In Rec. 2, however, the DNA flow histogram demonstrated an additional tetraploid peak (DI 1.95), whereas the karyotype showed merely 39~45 chromosomes. This might be the result of a growth advantage in tissue culture of cells with low chromosome numbers, or a sampling error with regard to which part of the tumor had been cultured.

TP53 mutations found are depicted in Table 2. A possibly causative mutation (Arg156-Pro) was found in the initial low-grade tumor, which persisted to Rec. 1b, the more malignant form of the first recurrence. This exon 5 mutation resulted in a nonconserved amino acid change within the DNA binding domain of the p53 protein. As depicted in Fig. 4, this mutation occurred hemizygously/homozygously in Rec. 1b, but heterozygously in the primary tumor and Rec. 2. This may be explained by

contamination from normal cells. An additional, possibly noncausative intronic mutation (intron 9) was found in the primary tumor and Rec. 1b. This mutation did not reappear after radiotherapy. According to the NetGene2 prediction program (Hebsgaard et al., 1996), this mutation does not affect the splicing of intron 9. In Rec. 1a, no causative exon 5 mutation or intron 9 mutation was found. However, an additional possibly causative mutation (Thr211-Ile) in exon 6 of the *TP53* gene resulting in a nonconserved amino acid change was found, suggesting that it may have arisen as an independent tumor in this patient. After radiotherapy (Rec. 2), the causative exon 5 mutation was observed, the intron 9 mutation was lost, and an additional intron 2 mutation involving a one-base-pair insertion, and presumably having no effect on the p53 protein, was also found. Unfortunately, Rec. 3 did not give any interpretable results.

Discussion

Astrocytomas in children are regarded as benign tumors and, after resection, no radiation therapy is given. It is known that after a total resection, recurrences hardly occur. The recurrence of tumor in low-grade astrocytomas is at the primary site or, when radiation therapy is given, in the area of the field of radiation (Dirks et al., 1994). Clinical parameters are important when considering the risk of recurrence in a patient with a low-grade astrocytoma, and regulation of cell proliferation and genetic abnormalities also may be of importance to expectancy of the survival of the patients (Nakamura et al., 1997).

Table 2. *TP53* gene analysis results

Sample	<i>TP53</i> mutation	Exon/intron
Primary tumor	Arg156-Pro +4bp; c-t	5 Intron 9
Rec. 1a	Arg156-Pro +4bp; c-t	5 Intron 9
Rec. 1b	Thr211-Ile	6
Rec. 2	Arg156-Pro -32bp; ins c	5 Intron 2

Abbreviations: +bp, base pairs from splice site donor site; -bp, base pairs from splice site acceptor site.

In our patient, with a long clinical history, the first tumor resection material showed 2 different components, one with low cellularity and one with increased cellularity and pleomorphism, although with insufficient evidence for full malignancy, and both with low MIB LI. The tumor rapidly progressed to a full blown malignancy with high mitotic activity and high MIB LI. The karyotype after radiotherapy was very complex, with at least 3 different clones, together with multiple balanced chromosomal rearrangements; whereas in the first recurrence, before radiotherapy, fewer different clones and no balanced chromosomal abnormalities could be found. It is known that therapeutic irradiation can induce solid tumors or perhaps change the character of already existing tumor tissue. Dirks et al. (1994) described 6 children who developed anaplastic astrocytomas after receiving radiation therapy for low-grade astrocytomas. Chauveinc et al. (1997) concluded that the presence of multiple chromosomal rearrangements, principally of the balanced type, and multiclonality strongly suggest the radiation-induced nature of 3 meningiomas in a single patient. So these 2 characteristics—multiclonality and high frequency of balanced chromosomal rearrangements—might be the result of radiation-induced changes in the already existing tumor in this patient.

Pediatric low-grade astrocytomas tend to yield normal karyotypes or only numerical chromosomal aberrations, whereas most complex karyotypes with structural abnormalities are found in more aggressive tumors like anaplastic astrocytomas and glioblastomas multiforme (Bhat-tacharjee et al., 1997; Biegel, 1997; Bigner et al., 1997; Fujii et al., 1994; Griffin et al., 1992; Karnes et al., 1992; Neumann et al., 1993; Sainati et al., 1996; Thiel et al., 1993; Wernicke et al., 1997). Chromosome 1 abnormalities are frequently seen in pediatric tumors of the CNS (Douglass et al., 1985; Sainati et al., 1996; Thiel et al., 1993). Sainati et al. (1996) suggested a possible association between chromosome 1 rearrangements and very aggressive childhood CNS tumors, regardless of the primary tumor histology. They also found complex karyotypes and pseudopolyploidy frequently associated with a poor prognosis.

As to a possible relation between prognosis and mutation of the *TP53* gene, according to the literature, *TP53* gene mutations appear to be an early event in the genesis of astrocytomas, at least in adults. Sidransky et al. (1992) suggested that mutation of *TP53* is an important step in

Fig. 4. DGGE aberrant banding pattern for the possibly causative *TP53* mutation occurring in exon 5 of the *TP53* gene. In both A and B, the DGGE banding pattern is representative of a control (lane 1), Rec. 1a (lane 2), and primary tumor (lane 3). In A, Rec. 1a appears as a hemizygous/homozygous single DGGE band. In B, Rec. 1a was mixed with control DNA before heteroduplexing and therefore gave the same banding pattern as heterozygous primary tumor.

the progression from low- to high-grade astrocytomas in adults. However, mutations of this gene are rare in both pilocytic and low-grade astrocytomas of childhood (Felix et al., 1995; Litofsky et al., 1994; Orellana et al., 1998), and there are hardly any data about progression of such tumors in relation to *TP53* mutation. Pollack et al. (1997) found *TP53* mutation or p53 immunopositivity in high-grade pediatric astrocytomas of the brainstem to be associated with poor survival. In low-grade adult astrocytomas, p53 immunopositivity seems to be related to a worse prognosis (Chozick et al., 1994; Iuzzolino et al., 1994). A previous study by Dirven (1998), enlarged to include a series of 20 pilocytic (World Health Organization grade I) and 2 low-grade (World Health Organization grade II) astrocytomas, showed *TP53* probably to be involved in these tumor types (Hayes et al., 1999b). The 4 tumors described in that series of patients with progression of residual tumor after postoperative follow-up, none of which had a *TP53* mutation, were all of the pilocytic type. Both low-grade astrocytomas (one with and one without a *TP53* mutation) were totally resected and, therefore, progression could not be assessed. A relationship with tumor behavior thus remains unclear. In retrospect, the unexpected aggressive behavior of the low-grade, nonpilocytic astrocytoma described in this study might have been predicted from the highly abnormal karyotype, together with the clonal abnormality of chromosome 1, found before radiation therapy.

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