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Molecular genetic alterations in pleomorphic xanthoastrocytoma

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Abstract Pleomorphic xanthoastrocytoma (PXA) is a low-grade glioma that may recur as a malignant diffuse astrocytoma such as glioblastoma (GBM). While the molecular genetic basis of diffuse astrocytomas has been studied extensively, PXAs have not been analyzed in detail. We, therefore analyzed DNA from archival primary and recurrent PXAs from eight patients (three grade II PXAs without recurrence, one grade II PXA with recurrence as grade II PXA, two grade II PXAs with progression to GBM, and two grade III anaplastic PXAs with recurrence as grade III anaplastic PXA or GBM) for genetic changes associated with diffuse astrocytomas. Single-strand conformation polymorphism analysis of p53 exons 5–8 revealed migration shifts in two cases, one primary PXA without recurrence and one recurrent grade II PXA in which the primary tumor did not show a shift. DNA sequencing showed two missense mutations in codons 220 (exon 6) and 292 (exon 8), respectively, mutations which have not been previously noted in astrocytomas. Differential polymerase chain reaction analysis demonstrated epidermal growth factor receptor gene amplification in only one tumor, a GBM without allelic loss of chromosome 10 that was the second GBM recurrence of an initial grade II PXA. Loss of heterozygosity studies on tumors from five patients, using three microsatellite polymorphisms on chromosome 10q and three on chromosome 19q, did not disclose allelic loss in any recurrent tumor. These findings suggest that the genetic events that underlie PXA formation and progression may differ significantly from those involved in diffuse astrocytoma tumorigenesis.

Key words Astrocytoma · Epidermal growth factor receptor · Glioma · p53 · Loss of heterozygosity

Dedicated to Dr. John J. Kepes

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Introduction

Pleomorphic xanthoastrocytoma (PXA) is a rare glioma, which usually occurs in the superficial cerebral hemispheres of children and young adults [7, 8, 27]. The pathological features include a mixture of pleomorphic, foamy and spindle-shaped cells, a reticulin network around individual tumor cells, immunohistochemical positivity for glial fibrillary acidic protein (GFAP), involvement of the leptomeninges, and frequently a cystic component. Although a controversy centered on the glial versus mesenchymal nature of this tumor [9, 23], almost all authorities now regard the PXA as being an astrocytic neoplasm [10].

Most PXAs, despite their pleomorphic histological appearance, have a relatively favorable prognosis. Post-operative follow-up periods of up to 18 years without recurrence have been reported [22]. Some cases of histologically typical PXA, however, may transform into a malignant glioma, usually a glioblastoma, with a correspondingly grim prognosis [1, 9, 19, 28]. Unfortunately, definite histological or molecular genetic features that herald the eventual malignant transformation of PXA have not been identified to date. The appropriate post-operative therapy of PXA is therefore uncertain, and clinical and radiological follow-up at short intervals has been recommended [26, 29].

Table 1 Clinical and molecular genetic data in eight patients with pleomorphic xanthoastrocytoma (*P* primary tumor, *R1, R2, R3* first, second and third recurrence, *PXA (II)* pleomorphic xanthoastrocytoma, *PXA III* anaplastic PXA, *A III* anaplastic astrocytoma, *GBM* glioblastoma, *NER* no evidence of recurrence after surgical resection, *yrs* years, *mths* months, *PCR* polymerase chain reaction, *EGFR* epidermal growth factor receptor, – no abnormal result of PCR or PCR-single-strand conformation polymorphism assay, + presence of p53 mutation or EGFR amplification, *n* no amplification product was obtained in the PCR reaction)

Case	Age	Sex	Diagnosis	Localization	Follow-up	p53	Mutation in Exon				EGFR amplification
						5I	5II	6	7	8	
1	27	f	PXA II	Temporal	NER 7 yrs	–	–	+	–	–	–
2	25	m	PXA II	Temporal	NER 6 yrs	–	–	–	–	–	–
3	43	m	PXA II	Temporal	NER 3 yrs	–	–	–	–	–	–
4-P	8	f	PXA II	Parietal	Recurrence 5 yrs	–	–	–	–	–	–
4-R1	13		PXA II		NER 5 yrs	n	n	–	n	+	–
5-P	3	f	PXA II	Temporal	Recurrence 3 yrs	n	n	–	n	–	n
5-R1	6		A III		Recurrence 2 yrs	–	–	–	–	–	–
5-R2	8		GBM		Recurrence 1 yr						
5-R3	9		GBM		Death 6 mths	–	–	–	–	–	–
6-P	19	f	PXA II	Temporal	Recurrence 2 yrs	–	–	–	–	–	–
6-R1	21		GBM		Recurrence 2 yrs	–	–	n	–	n	–
6-R2	23		GBM		Recurrence 8 mths	–	–	–	–	–	+
7-P	18	m	PXA III	Temporal	Recurrence 8 mths	–	–	–	–	–	–
7-R1	19		PXA III		Recurrence 2 yrs	–	–	–	–	–	–
7-R2	20		GBM		NER 1 month						
8-P	14	f	PXA III	Temporal	Recurrence 10 yrs	–	–	–	–	–	–
8-R1	24		PXA III		No follow-up	–	–	n	–	–	–

No material was available from tumors 5-R2 and 7-R2

The molecular genetic correlates of malignant progression have been extensively examined in the more common diffuse astrocytomas (reviewed in [16]). Approximately one-third of diffuse grade II-IV astrocytomas harbor mutations of the p53 tumor suppressor gene and/or allelic loss of chromosome 17p, which probably represent the earliest detectable genetic alterations during astrocytoma progression. Grade III tumors (anaplastic astrocytomas) have often acquired inactivations of the retinoblastoma susceptibility gene on chromosome 13q (30% of tumors) and of putative tumor suppressor genes on chromosomes 9p (20%) and 19q (45%). Transformation into grade IV tumors (glioblastomas) is associated with allelic loss of chromosome 10 (70%) and amplification of the epidermal growth factor receptor (EGFR) gene (35%). To our knowledge, however, PXAs have not been subject to extensive genetic analyses.

We have examined eight cases of PXA, both with and without subsequent recurrence or malignant transformation, for molecular genetic abnormalities. To gain insight into the genetic processes underlying the unpredictable outcome associated with this tumor, we specifically analyzed whether PXAs and their malignant recurrences show changes similar to those of diffuse astrocytomas.

Materials and methods

Tumor samples were examined from three patients without recurrence (cases 1–3) and from five patients suffering from one or several recurrences (cases 4–8). The basic clinical data are listed in Table 1. Further details of the clinical history and the neuroradiological appearance of cases 2, 4, 5, 6 and 7 have been described elsewhere (cases 4, 1, 3, 5 and 6 in [26]).

The tumors were obtained by biopsy and fixed in either formalin or Somogyi's solution (4% paraformaldehyde, 0.05% glutaraldehyde, 15% picric acid, Sörensen's phosphate buffer pH 7.3). Paraffin blocks were available from all primary and recurrent tumors (except for tumors 5-R2 and 7-R2, see Table 1). The pathological diagnoses (Table 1) were made by two neuropathologists (W.P. and D.N.L.) according to the WHO Classification [10] using hematoxylin-eosin (H&E), reticulin and immunohistochemical studies for GFAP.

For DNA extraction, representative tumor areas without cerebral or leptomeningeal tissues were labeled on H&E sections. These areas were scraped from adjacent 6-μm-thick non-stained paraffin sections into microcentrifuge tubes. A total of approximately 1-cm^2 tissue was used per tumor, corresponding to one to three slides. After treatment with xylene and graded ethanols and subsequent centrifugation steps, the tissue was digested in a solution containing 1 mg/ml proteinase K, 1 mM EDTA, 0.5% Tween 20 and 10 mM TRIS-HCl, pH 8.0, for 48 h at 50°C. The specimens were boiled for 10 min and centrifuged at 15,300 *g* for 5 min. The supernatant was subjected to ethanol precipitation, resuspended in sterile water and used for polymerase chain reaction (PCR). Because of the retrospective nature of this study, constitutional DNA was not available.

The presence of EGFR gene amplification was investigated using differential PCR, as described [17]. This technique is able to reveal EGFR gene amplification in archival specimens from gliomas with high sensitivity and specificity [6, 17]. Briefly, three duplex PCR reactions were performed for each tumor to amplify a 110-bp fragment of the EGFR gene as well as two reference DNA fragments of 82 bp and 150 bp from the interferon-γ (IFN-γ) gene using two primer pairs in each reaction, i.e. IFN-γ 150 and IFN-γ 82, IFN-γ 150 and EGFR 110, and EGFR 110 and IFN-γ 82 [20]. Absent or markedly diminished IFN-γ bands in the presence of the EGFR band indicate genomic EGFR amplification, because these EGFR sequences are preferentially PCR-amplified. Thirty-five PCR cycles with an annealing temperature of 52°C were per-

formed. The products were resolved on 2% agarose gels and stained with ethidium bromide.

The p53 gene was studied using single-strand conformation polymorphism analysis (SSCP). Exons 5–8 of the p53 gene were amplified from tumor DNA using published primers and PCR conditions [25]. Because of the large size of exon 5, two PCR reactions amplifying different parts of this exon were performed. Amplification products were denatured (2 min at 95° C), placed on ice, and separated on 6% non-denaturating polyacrylamide gels containing 10% glycerol for 13–16 h at 8 W. For autoradiography, dried gels were exposed for 1–3 h at –80° C. SSCP shifts were confirmed by a second identical assay. Asymmetric PCR was performed to obtain single-stranded products of those exons that showed an abnormal migration pattern on SSCP. The primers were the same as for PCR-SSCP. Dideoxy sequencing was performed using the Sequenase kit from USB. Both strands of exons with mobility shifts were sequenced twice.

$(CA)_n$ dinucleotide repeat assays for detection of allelic heterozygosity was performed as described [18]. The following $(CA)_n$ repeat loci were amplified using PCR: D10S88, D10S109 and D10S169 on the long arm of chromosome 10 [17], and D19S112, APOC2 and BCL3 on the long arm of chromosome 19 [24]. Because constitutional tissues were not available for study, our loss of heterozygosity studies were limited. Therefore, only cases with material available from at least two resections were studied (cases 4–8).

Results

All PXAs showed the typical histological features, including pleomorphic and multinucleated cells, foamy cells, spindle-shaped cells, granular bodies, perivascular chronic inflammatory cells, a reticulin network around individual tumor cells, and positivity for GFAP in a variable fraction of cells, but no necrotic areas [8, 10]. Very rare mitoses [less than 1 per 10 high-power fields (HPF)] were seen in tumors 5-P and 6-P. The tumors from patients 7 and 8 contained a markedly increased number of mitoses (from 1 per 3 HPF to 3 per HPF) and mild endothelial/pericytic hyperplasia, but no other indications of malignancy; these tumors were, therefore, tentatively graded as III, although criteria for the grading of PXA as II or III have not been published [11]. In addition to the usual circumscribed growth pattern of PXA, focal diffuse invasion of tumor cells into brain tissue was seen in grade II PXAs 1, 4-P (but not 4-R1) and 6-P, and this feature was more pronounced in all grade III PXAs. The first recurrence in patient 5 (tumor 5-R1) was an anaplastic astrocytoma (without PXA features) that was adjacent to, but well delineated from, a smaller area corresponding to grade II PXA. All glioblastomas showed the typical histological appearance with nuclear pleomorphism, necrotic areas, endothelial/pericytic hyperplasia and mitoses, but they did not contain unequivocal PXA areas. In contrast to a previous report [9], the glioblastomas were not predominantly composed of small tumor cells; instead, in cases 5 and 6 they contained astroblastomatous areas and prominent perivascular pseudopapillary formations.

PCR of exons 5–8 of the p53 gene revealed amplification products in 66/75 assays (88%), corresponding to previously published success rates using routinely processed brain tumor materials [17]. On SSCP analysis, a migratory shift was observed in two tumors (1 and 4-R1; Table 1, Fig. 1). Sequencing of these two PCR products revealed a missense mutation in both cases: TAT to TGT (tyrosine to cysteine, Fig. 2) in codon 220 (exon 6) and AAA to CAA (lysine to glutamine) in codon 292 (exon 8). Both tumors had lost the remaining wild-type p53 allele, as evidenced by loss of the wild-type band on direct sequencing. Interestingly, the primary tumor of patient 4 did not show the mutation in exon 8. The histological features of the primary and recurrent tumors of patient 4 were similar.

Differential PCR for EGFR amplification was evaluable in 14/15 tumors (93%). Selective PCR amplification only of the EGFR sequences but not the control sequences, indicating EGFR amplification of tumor DNA, was seen

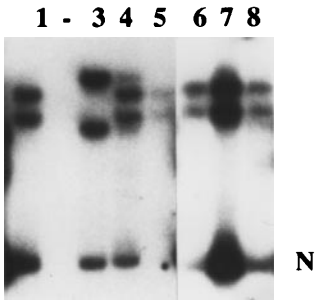

Fig. 1 Single strand conformation polymorphism analysis of exon 6 of the p53 gene shows shifted single-strand bands in *lane 3*, case 1. (*N* = nondenatured double-stranded products, – no DNA lane)

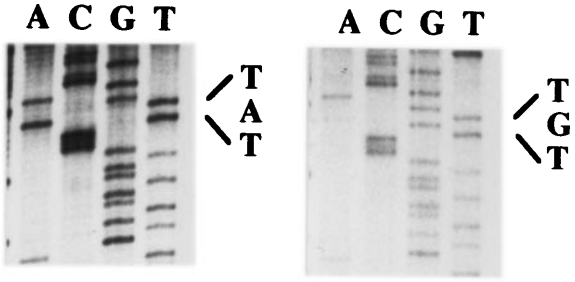

Fig. 2 DNA sequencing of exon 6 of the p53 gene in case 1 *(right panel)* demonstrates a TAT to TGT missense mutation (tyrosine to cysteine) in codon 220, when compared with the wild-type sequence *(left panel)*

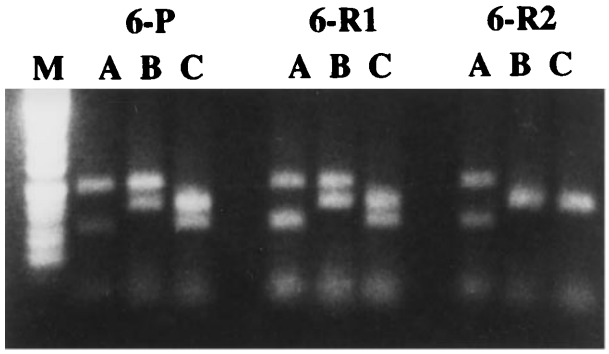

Fig. 3 Differential polymerase chain reaction analysis reveals epidermal growth factor receptor (EGFR) gene amplification in the second recurrence of case 6 (6-R2), as evidenced by the loss of the upper and lower interferon-γ (IFN-γ) bands in *lanes 6-R2 B* and *C*, respectively. In each panel, *lanes A* show the upper and lower IFN-γ bands; *lanes B*, the upper IFN-γ and the EGFR bands; *lanes C*, the lower IFN-γ and the EGFR bands (*M* size marker DNA)

in only one tumor, a glioblastoma that was the second recurrence of a grade II PXA in patient 6 (tumor 6-R2, Fig. 3, Table 1). The first recurrence, also a glioblastoma, did not show EGFR amplification (Fig. 3). The histological features did not differ between the first and the second recurrence.

Loss of heterozygosity studies using three microsatellite polymorphisms on chromosome 10q and three on chromosome 19q, performed on 12 tumors from the five patients suffering from recurrence (patients 4–8), were informative in 49 of 72 assays. No allelic loss was disclosed in any recurrent tumor.

Discussion

p53 mutations have been detected in 20–40% of diffuse astrocytomas of grades II-IV, but are rare in other brain tumors [14, 21]. We found missense mutations at codons 220 and 292 of the p53 tumor suppressor gene in two grade II PXAs which did not progress to malignant gliomas. Although our study comprised only eight patients, the relative frequency of 25% of PXAs with p53 mutation (two out of eight patients) corresponds more closely to diffuse astrocytomas than to grade I pilocytic astrocytoma or other brain tumors. On the other hand, the two p53 mutations detected in PXAs are unusual for four reasons. First, they are located outside the conserved domains of the p53 gene, whereas in diffuse astrocytomas approximately two-thirds of mutations occur in the conserved domains II, III, IV and V [15]. Second, mutations of codons 220 and 292 have not been reported in brain tumors so far. In the EMBL database which covers 2640 p53 gene mutations [5], missense mutations at codon 220 have been described in 36 cases of carcinoma and lymphoma, with the TAT to TGT change, also found in our case 1, apparently being the most common mutation at this codon. Only two tumors in this database (stomach and thyroid carcinoma) showed missense mutations of codon 292, which were different from the mutation found in our case 4-R1. Third, both PXA mutations involve A : T pairs, whereas in diffuse astrocytomas 80% of the mutations occur at G : C pairs [15]. Fourth, one mutation was present in the benign recurrence of a PXA, but not in the primary PXA resected 5 years before. Acquisition of a p53 mutation in a recurrent tumor is rare in diffuse astrocytomas, and p53 mutations have been interpreted as the earliest detectable genetic aberrations in astrocytoma tumorigenesis [2, 12]. Our data suggest that p53 mutations may occur after establishment of a PXA, but are not associated with malignant progression of PXA.

Amplification of the EGFR gene occurs in about 35% of glioblastomas, and was observed in the present study in one of two glioblastoma cases which had developed from grade II PXAs. This observation is unusual for two reasons. First, the amplification was present only in the second recurrence and not in the first recurrence, which was also a glioblastoma. In contrast, in glioblastomas non-related to PXAs, primary and recurrent tumors show the same pattern of EGFR gene amplification [3, 4]. Second, the EGFR gene amplification in the present case occurred without detectable loss of heterozygosity for chromosome 10, whereas EGFR gene amplification is associated with allelic loss of chromosome 10 in most [13] or even all [3] glioblastomas not related to PXA. Loss of heterozygosity for chromosomes 10q and 19q, present in 70% of glioblastomas and 45% of anaplastic astrocytomas, respectively, was not found in the two PXA-associated glioblastomas.

In conclusion, both diffuse astrocytomas and PXAs may show p53 gene mutations, and EGFR gene amplifications may occur in glioblastomas with or without previous PXA. On the other hand, the timing of p53 and EGFR gene alterations, as well as the type of p53 mutations, may differ between diffuse astrocytoma and PXA, suggesting that the genetic events that underlie PXA formation and progression may differ somewhat from those involved in diffuse astrocytoma tumorigenesis. Additional tumors of this rare entity must be examined to gain a better understanding of the role of molecular genetic aberrations in PXA formation and progression.

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