Molecular Characterization of Areas with Low Grade Tumor or Satellitosis in Human Malignant Astrocytomas

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ABSTRACT

Malignant astrocytomas often display histopathological heterogeneity. In the present study, we have molecularly characterized different areas within 4 such tumors to determine whether the tissue heterogeneity can be explained by differences in DNA constitution. Two tumors contained low grade areas, and the other 2 had areas with satellitosis. The tumors were examined for loss of heterozygosity with markers from chromosomes 9p, 10, and 17p and for amplification of the epidermal growth factor receptor gene. In each case, the high grade portion of the tumor displayed at least one of these structural alterations. However, identical alterations were found in the associated low grade or satellitosis areas of each tumor. Our data suggest that: (a) genetic alterations associated with tumor progression already occur in histopathologically low grade areas of high grade astrocytoma; (b) satellitosis associated with a high grade astrocytoma has to be considered as part of that tumor; and (c) tissue heterogeneity within a high grade astrocytoma is not a consequence of differences in DNA constitution at the loci that were examined.

INTRODUCTION

Heterogeneity is a frequent finding in human malignant astrocytomas. Based on morphological criteria, there is heterogeneity of type and grade. Within a high grade tumor, areas with low grade tumor or with reactive gliosis can be found (1, 2). Heterogeneity has also been observed in the expression of tumor antigens (3-5) and in the karyotype of subpopulations of cells derived from malignant astrocytomas (6).

Molecular analyses have been performed on pure high and low grade astrocytomas. LOH studies have shown that one copy of chromosome 10 is frequently lost in high grade astrocytomas but not in low grade astrocytomas (7-9). Recently, frequent deletion of chromosome 9p, including the α- and β-IFN genes, was detected in intermediate and high grade astrocytomas but not in tumors with a low malignancy grade (10). LOH for chromosome 17p loci has been reported for low as well as high grade astrocytomas (9, 11-13). Point mutations in the p53 gene on 17p have been found in 4 of 5 high grade astrocytomas (14), emphasizing the important role of this tumor suppressor gene in the development of astrocytomas. Finally, amplification of the EGFR gene was found in 40-50% of high grade astrocytomas but not in low grade tumors (7, 15).

Except for one study (16), the separate molecular analysis of tissue samples of histopathologically heterogeneous malignant astrocytomas has not been reported so far. In that study, it was found that LOH for chromosome 10 markers occurred in different grades of parts of 3 malignant astrocytomas, indicating that there was no heterogeneity with respect to this particular chromosome.

To determine whether there is a correlation between histological grading and DNA constitution, we molecularly characterized 4 malignant astrocytomas of mixed composition. Two had distinct low grade portions of sufficient volume for DNA analysis. The other 2 contained extensive areas of reactive gliosis with satellitosis. Satellitosis is characterized by the typical grouping of neoplastic astrocytes around neurons when the tumor infiltrates the gray matter (17).

The different samples of the tumors were analyzed for loss of chromosome 17p and for genetic alterations associated with tumor progression, i.e., loss of chromosomes 9p and 10 and EGFR gene amplification. Here we report that the molecular changes found in the high grade areas of the respective tumors were already present in their associated low grade or satellitosis areas. From this we conclude that histopathologically heterogeneous astrocytomas have the genetic features of their phenotypically most malignant area.

MATERIALS AND METHODS

Tumor Specimens. Tumors were obtained during brain surgery. For routine diagnostic histology, one part of each tumor was embedded in paraffin and 6-μm sections were stained with hematoxylin and eosin, Gomori’s reticulin, and phosphotungstic acid hematoxylin. The other part of the tumor was quickly frozen in liquid nitrogen and stored at −70°C until examination. Grading of the areas within malignant astrocytomas was done using the grading system of Kernohan and Sayre (18). Grade I-II corresponds to the WHO classification astrocytoma, grade III to anaplastic astrocytoma, and grade IV to glioblastoma multiforme, the key difference between grade III and IV being the presence of necrosis in grade IV (19). Areas with different grading were selected on the basis of the histological examination with hematoxylin and eosin of 6-μm frozen sections. In case of tumor S4 (see “Results”), samples were taken from the center and peripheral and peritumoral parts of the tumor prior to cooling in liquid nitrogen. Only histopathologically pure tissue samples of sufficient volume were taken for DNA analysis.

DNA Isolation and Southern Blot Analysis. Tumor tissue samples were homogenized in 75 mM NaCl, 25 mM EDTA. Next, the suspensions were incubated for 16 h at 37°C in the presence of 1% SDS and 150 μg/ml proteinase K. Lysates were then extracted twice with phenol and twice with chloroform before precipitating the DNA with 1.5 volumes of ethanol and 0.1 volume of 3.0 M NaAc, pH 4.8. Samples were suspended in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA and quantitated spectrophotometrically. Venous peripheral blood samples, taken prior to surgery, were incubated for 20 min at 0°C with 3 volumes of 0.15 M NaHCl, 10 mM KHCO3, 0.1 mM EDTA. The nuclear fraction was pelleted; suspended in 75 mM NaCl, 25 mM EDTA; and processed as described for the tumor tissue samples.

Samples of approximately 5 μg blood or tumor DNA were digested with the appropriate restriction enzymes under conditions recommended by the supplier (Boehringer Mannheim), fractionated with agarose gel electrophoresis, and transferred to nylon membranes (Gene Screen Plus; New England Nuclear) in 0.5 N NaOH, 1.5 M NaCl. Inserts of probes were recovered from low melt temperature agarose (Bio-Rad) and 32P-labeled by the random oligonucleotide primer procedure (20). Probes were boiled for 10 min, if necessary in the presence of excess sheared and denatured human DNA to block repetitive sequences, and hybridized for 16 h at 65°C to the nylon membranes in intercalating dye.
0.5 mM NaHPO₄, pH 6.8, 7% SDS, 1 mM EDTA, containing 50 μg/ml herring sperm DNA. After hybridization, the membranes were washed 3 times, 5 min each time, at 65°C in 0.04 M NaHPO₄, pH 6.8, with 1% SDS, 0.1% SDS, and without SDS, respectively, and exposed to Kodak X-Omat film for 1–5 days at −70°C with 2 intensifying screens.

**Probes.** Loss of heterozygosity for loci (between brackets) on chromosomes 9p, 10, and 17p was analyzed by using the following probe/enzyme combinations: βIFN/MspI or SacI (IFNB1), DR6/HindIII or SacI (D9S3), pMHZ15/MspI (D10S17), H4 IRBP/BglII or MspI (RBP3), pMCX2/PruvII (D10S15), p9-12/PruvII (D10S5), pTB10.163/MspI (D10S22), pTB10.171/PruvII (D10S19), pTHH54/MspI (D10S14), p1–10/PruvII (D10S4), pEF75/PruvII (D10S25), pYNN22.1/BamHI (D17S58), pYNH37.3/MspI or PruvII (D17S28). LEW504/MspI (D17S68), LEW502/BglII (D17S66), p10–5/MspI (MYH2), LEW503/MspI (D17S67), UC10–41/MspI or PruvII (D17S71), and LEW301/BglII or PruvII (D17S58).

**IFNB1 gene dosages** were determined as follows. Southern blots of EcoRI-digested paired leukocyte and tumor DNAs were hybridized with βIFN (locus IFNB1) and subsequently, after removal of the probe, with pDP34 ( locus DXYS11) (10). The latter probe failed to reveal LOH in a large number of gliomas (10) and was, therefore, used as an internal reference marker. The autoradiographic signals obtained were quantified with a Phosphorimager (Molecular Dynamics) using Imagequant Software v3.0. The normalized IFNB1 gene dosage was calculated by using the formula (10):

\[
\frac{\beta\text{IFN}(t)/\text{DP34}(t)}{\beta\text{IFN}(n)/\text{DP34}(n)} \times 2
\]

in which βIFN represents the signal obtained with probe βIFN and DP34 the signal obtained with probe DP34 in tumor (t) and corresponding leukocyte (n) DNA, respectively. A value of 2.00 represents the normalized IFNB1 gene dosage of a tumor without LOH and 1.00 of a tumor with LOH. In a tumor with LOH, a 10% contamination with normal cells would result in a reduction of this value to 1.1. This was considered to be acceptable (cf. Ref. 10).

Amplification of the EGFR gene was measured by densitometric scanning of bands, ranging in size from 1.3 to 8.5 kilobases, obtained after hybridizing probe pE7 to Southern blots of BglII-digested DNAs.

Physical location and other characteristics of each probe are from References 21–23.

**RESULTS**

In an ongoing study aimed at the molecular characterization of tumors of astrocytic origin, we encountered 4 cases in which the different areas of a heterogeneous high grade tumor could be separated in tissue blocks large enough for DNA extraction. Special care was taken to avoid contamination of the various tissue samples with neighboring tissues of different grading. DNA was extracted from the tissue samples and analyzed for LOH by Southern blot analysis with DNA markers of chromosomes 9p, 10, and 17p using leukocyte DNA of each patient as constitutional DNA. Possible chromosome 9p deletions were also inferred from the IFNB1 gene copy number in each tissue sample (cf. Ref. 10). Finally, we determined whether the EGFR gene was amplified in the various tumor specimens.

**Tumor S1.** This malignant astrocytoma was histopathologically classified as a grade III tumor (anaplastic astrocytoma) containing areas of reactive gliosis with satellitosis (see Fig. 1A). IFNB1 dosage and LOH analyses, summarized in Tables 1 and 2, indicate that both copies of chromosomes 9p and 10 are present in the 2 tumor samples. On chromosome 17p, heterozygosity was maintained for the proximal loci D17S58 and D17S71, but lost for D17S66 in chromosomal band 17p13.1. The p53 tumor suppressor gene is also contained within this band (21). Almost complete loss of the large allele of D17S66 in both samples is displayed in Fig. 1B. The EGFR gene was not amplified in the 2 tissue samples (Table 1).

**Tumor S3.** The tumor was histopathologically classified as a grade III tumor (anaplastic astrocytoma) containing areas of reactive gliosis with satellitosis (see Fig. 1A). IFNB1 dosage and LOH analyses, summarized in Tables 1 and 2, indicate that both copies of chromosomes 9p and 10 are present in the 2 tumor samples. Chromosome 17p, heterozygosity was maintained for the proximal loci D17S58 and D17S71, but lost for D17S66 in chromosomal band 17p13.1. The p53 tumor suppressor gene is also contained within this band (21). Almost complete loss of the large allele of D17S66 in both samples is displayed in Fig. 1B. The EGFR gene was not amplified in the 2 tissue samples (Table 1).

**Tumor S4.** For removal of the tumor, the patient underwent lobectomy of the right frontal lobe. For that reason, normal brain was available in addition to tumor material. The tumor was defined as a grade IV astrocytoma (glioblastoma multiforme) with areas of lower grading and regions with satellitosis. Pure tissue samples could be obtained from the grade IV part and from an area containing reactive gliosis with satellitosis (see Fig. 1A). Only 2 chromosome 10 markers, D10S5 and D10S22, were informative in this patient. We detected loss of heterozygosity with the 2 markers in the grade IV as well as the satellitosis section of the tumor but not in normal brain. Almost complete loss of the large allele of D17S66 in both samples is displayed in Fig. 1B. The EGFR gene was not amplified in the 2 tissue samples (Table 1).

**DISCUSSION**

Histopathological heterogeneity is one of the hallmarks of malignant tumors. According to Nowell's (25) hypothesis of tumor evolution, this might be explained by assuming that the different cell populations in a malignant tumor are derived from the same ancestor cell but differ by the genetic changes that were acquired during progression of the tumor.

In astrocytomas, loss of chromosomes 9p and 10 and amplification of the 9p marker D9S3 in the 2 tissue samples (see Fig. 1B). We found LOH with nearly all chromosome 10 markers in both tissue samples. A representative example, showing loss of the large allele of marker D10S19, is shown in Fig. 1B. The only exception was D10S25, located in the distal part of the long arm of chromosome 10. The 2 tumor specimens remained heterozygous with this marker. Partial loss of chromosome 10 in malignant astrocytomas has been reported by others (8, 24). Heterozygosity was maintained for loci on 17p. The LOH data for this tumor are summarized in Table 2. The EGFR gene was in both tumor samples amplified to the same level, i.e., more than 10 times (Table 1).

**Tumor S2.** This grade IV astrocytoma (glioblastoma multiforme) also contained areas of low grade tumor (grade I-II; Fig. 1A). From the data presented in Tables 1 and 2, we conclude that the 2 copies of chromosomes 9p and 10, respectively, are still present in the high grade and low grade sample of the tumor. Heterozygosity was lost for D17S5, in the telomeric region of 17p and D17S58, in the centromeric region of 17p, and for all other informative loci between these loci. Loss of the small allele of D17S55 and D17S58 in the 2 samples is shown in Fig. 1B. Both tissues retained heterozygosity for several markers of the long arm of chromosome 17 (data not shown). These data strongly suggest that the whole short arm of chromosome 17, including the p53 tumor suppressor gene, was lost in both tissue samples. The EGFR gene was not amplified in the 2 areas of the tumor that we examined (Table 1).

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MOLECULAR ANALYSIS OF HETEROGENEOUS ASTROCYTOMAS

Fig. 1. Histopathological (A) and restriction fragment length polymorphism (B) analysis of tissue samples from heterogeneous high grade astrocytomas. A, histological sections of the tissue samples of each tumor stained with H&E (×250). B, autoradiographs showing LOH analysis of the tumor tissue samples histopathologically characterized under A. Pairs of tumor tissue samples and corresponding leukocyte DNAs were digested with the indicated enzymes. The resulting fragments were separated on agarose gels, blotted to nylon filters, and hybridized with 32P-labeled probes that identify the indicated loci. Lengths of the restriction fragments are given in kilobase pairs. L, leukocytes; A, astrocytoma; Sat, satellitosis; N, normal brain.

Table 1  IFNB1 gene dosage and EGFR gene amplification in heterogeneous high grade astrocytomas

The IFNB1 gene copy number in the tissue sample of each tumor was deduced from the normalized IFNB1 gene dose value. Gene dosages were calculated as described in "Materials and Methods." These were 1.12 and 1.13 for the low and high grade portions of tumor S1 and ranged from 2.14 to 2.49 for the other tissue samples. EGFR gene amplification was determined by densitometric scanning of the signals obtained after hybridizing probe pE7 (identifying locus EGFR) to Southern blots of BglII-digested pairs of tumor tissue sample and corresponding leukocyte DNAs.

<table>
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<tr>
<th>Locus</th>
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<th>Tumor S1 Grade I, II</th>
<th>Tumor S1 Grade III</th>
<th>Tumor S2 Grade I, II</th>
<th>Tumor S2 Grade IV</th>
<th>Tumor S3 Grade I, II</th>
<th>Tumor S3 Grade IV</th>
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<th>Tumor S4 Grade IV</th>
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<td>EGFR</td>
<td>7p13-p12</td>
<td>+</td>
<td>+</td>
<td>+</td>
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</table>

* Sat, satellitosis; N, normal brain.
+ +, EGFR gene more than 10 times amplified; –, EGFR gene not amplified.

Tumor progression inasmuch as these events are not detected in pure low grade astrocytomas (7, 9, 10, 15). We wondered whether differences in DNA constitution at these chromosomal regions could possibly explain the differences in histopathological grading within malignant astrocytomas. In an earlier report (16), low grade areas within malignant astrocytomas were analyzed. It was found that in 3 of 3 tumors the differently graded regions all showed LOH for chromosome 10 loci. In the present study, we confirm these findings and extend them to the other
Table 2 LOH for loci on chromosomes 9p, 10, and 17p in heterogeneous high grade astrocytomas

<table>
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*—, Not informative, leukocyte DNA homozygous for the marker; 1,2, no LOH, both alleles retained in the tumor tissue sample; 1, LOH, loss of the small allele in the tumor tissue sample; 2, LOH, loss of the large allele in the tumor tissue sample.

Genetic alterations associated with tumor progression. In tumor S1, diagnosed as an astrocytoma grade III (anaplastic astrocytoma) with low grade areas, we found LOH for all but one chromosome 10 marker in a low as well as high grade tissue sample (Table 2). In addition, we found that one copy of chromosome 9p was deleted and that the EGFR gene was already amplified in the low grade portion of that tumor (Table 1). These observations demonstrate that the genetic alterations associated with tumor progression had already taken place in the low grade area of this high grade astrocytoma.

In case of tumor S2, one copy of chromosome 17p was lost in the low and high grade portion of that tumor (Table 2). Other possible differences were not found. Separate analysis of a carcinoma region within a colon adenoma revealed deletion of one p53 gene on chromosome 17p accompanying the transition of the benign to the malignant state in that tumor (26). Such a deletion cannot explain the histopathological differences in these tumors. However, additional genetic differences, which could possibly explain the histopathological differences, were not found. The histopathological differences might be caused by nongenetic influences, for instance by factors elaborated by neighboring normal brain cells. Alternatively, allelotyping of malignant astrocytomas (24) has identified additional loci on chromosomes 5q, 7p, 11p, 14q, and 15q as possible sites for tumor suppressor genes important in astrocytoma tumorigenesis. It will be of interest to determine whether there are differences in DNA constitution at these loci between differently graded areas within high grade astrocytomas.

The molecular analysis of the tumors S3 and S4 shows that genetic alterations in a high grade area of an astrocytoma are also present in a satellitosis part of that tumor (Tables 1 and 2). The large allele of D17S66 in the satellitosis area of tumor S3 and the large allele of D10S22 in the satellitosis area of tumor S4 are not completely lost, indicating that some normal material is still present (Fig. 1). These observations support the histopathological definition of satellitosis, being composed of neoplastic astrocytes infiltrating but not completely eliminating the normal cells in the gray matter. They also demonstrate that satellitosis has to be considered as an intimate part of a malignant astrocytoma. This may account for the fact that malignant astrocytoma is known to give relapse of tumor growth in almost all instances (27).

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