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Clonal Analysis of Human Meningiomas and Schwannomas[1]

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ABSTRACT

Meningiomas and schwannomas are two of the most common tumors of the human nervous system. To determine whether these tumors arise from a single cell or from multiple cells, we used molecular genetic techniques to study X chromosome inactivation in meningiomas and schwannomas isolated from females including one who had neurofibromatosis type 2. The tumors were also screened for loss of heterozygosity at several loci on chromosome 22 using polymorphic DNA markers. Among nine meningiomas, at least three of which showed loss of alleles on chromosome 22 and five of which retained heterozygosity for the chromosome 22 alleles examined, all nine tumors were monoclonal. Among eight schwannomas, at least seven of which retained heterozygosity for chromosome 22 loci, seven were monoclonal. We conclude that human meningiomas and schwannomas arise from a single cell.

INTRODUCTION

Meningiomas and schwannomas are two of the most common tumors of the human nervous system (1). They occur most often as solitary sporadic nonheritable tumors, but they may also occur in association with NF2,[3] an autosomal dominant genetic disorder.

Recent molecular genetic studies have documented the loss or alteration of chromosome 22 in most of the benign tumors associated with NF2, including meningiomas and acoustic neuromas (2–4). The formation of a tumor through loss or inactivation of a gene has been previously described in other tumors, for example, retinoblastoma (5) and Wilms' tumor (6, 7), and is consistent with a mechanism of tumorigenesis involving loss or inactivation of a tumor suppressor gene. Thus, in NF2, it is thought that tumors form only when the normal copy of the gene is deleted or altered leaving the inherited defective copy remaining. These observations have been extended to the general population since unilateral sporadic nonheritable acoustic neuromas (vestibular schwannomas) or meningiomas occurring in patients without NF2 may also be associated with loss or alteration of chromosome 22.

Knowledge of whether a tumor has a single or multiple cell origin may provide important information about its etiology and pathogenesis. A single cell origin suggests that the tumor arose from a single rare event such as a spontaneous somatic mutation. If, on the other hand, many cells participate in the origin of the tumor, they may have been influenced by a carcinogen or other humoral factor. The design of logical treatment strategies would differ in each instance.

Recently, Vogelstein *et al.* (8) described a molecular genetic approach to determine the clonal origin of tumors using DNA restriction length fragment polymorphisms of the X chromosome. This technique is based on three premises. First, according to the Lyon hypothesis, one of the X chromosomes in each cell is randomly inactivated early in the development of the female embryo and this inactivation pattern is inherited in a stable manner by the progeny cells. Second, genes on the active and inactive X chromosomes differ in the methylation of cytosine residues and these methylated cytosines are readily detected by certain restriction endonucleases. Third, the maternal and paternal X chromosomes contain normal variations that can be detected using DNA polymorphisms.

To carry out the clonal analysis, we used a probe that detects a polymorphism in the 5′ region of the X chromosomal gene HPRT. Cellular DNAs were digested with one endonuclease that distinguishes the maternal and paternal copies of the X-linked gene through a restriction fragment length polymorphism. The DNAs were also digested with the first enzyme plus a second endonuclease such as *Hpa*II that distinguishes inactive from active copies of the gene based on changes in methylation. If a tumor is monoclonal, the paternal copy of the gene will be cleaved by the second enzyme in a different manner from the maternal copy, since the paternal gene will be active in all cells or inactive in all cells. If the tumor is polyclonal, one-half of the cells will have an active paternal gene and one-half an active maternal gene, so that both gene copies will be equally affected by the second enzyme.

We have examined nine meningiomas and eight schwannomas from female patients. One of these tumors arose in a patient with NF2, while the others represented sporadic tumors appearing in the general population. In this report we present evidence that both meningiomas and schwannomas, irrespective of whether they have lost heterozygosity for chromosome 22 loci, are monoclonal in origin.

MATERIALS AND METHODS

After obtaining informed consent, tumor specimens were obtained at the time of surgery and frozen for later DNA analysis. Blood samples were also routinely obtained at the time of surgery to serve as normal tissue controls. Several meningiomas were also cultured for 1–3 weeks and then karyotyped as previously described (9).

High molecular weight DNA was extracted from peripheral blood leukocytes and from frozen pulverized tumor tissue by sodium dodecyl sulfate-proteinase K digestion followed by phenol and chloroform extractions (2). DNAs were digested with restriction endonucleases according to the recommendations of the supplier (New England Biolabs, Beverly, MA, or Boehringer Mannheim, Indianapolis, IN) or of Vogelstein *et al.* (10). The digested DNAs were resolved by agarose gel electrophoresis and transferred to nylon membrane filters (Hybond-N, Amersham, Arlington Heights, IL) by Southern blotting (11). DNA probes were labeled with [^{32}P]dATP by random oligonucleotide priming (12) and were hybridized to filters for 48 h at 65°C in 6× standard saline citrate (1× standard saline citrate = 0.15 M sodium chloride-0.015 M sodium citrate, pH 7.4), 1× Denhardt's solution (50× Denhardt's solution = 10 g/liter polyvinylpyrrolidone-10 g/liter bovine serum albumin-10 g/liter Ficoll-400), 0.3% sodium dodecyl sulfate, and 0.1 mg/ml salmon testis DNA. The filters were washed in 3–0.5× standard saline citrate at 65°C and exposed to X-ray film (Kodak XAR-

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[3] The abbreviations used are: NF2, neurofibromatosis, type 2; HPRT, hypoxanthine phosphoribosyltransferase.

5) with a Dupont Cronex intensifying screen at −80°C for 48 h. The intensities of DNA fragment bands were quantitated using a LKB Ultroscan XL densitometer. The HPRT probe DNA was a 700-base pair *Bam*HI/*Xho*I fragment derived from pHPRT800, generously provided by B. Vogelstein (10). Chromosome 22 probes included W21G (locus D22S24) (13), IGLV (14), BCRY11 (CRYB2) (15), pMS3–18 (D22S1) (16), W23C (D22S28) (13), W22D (D22S29) (13), and W24F (D22S23) (13).

RESULTS

Meningiomas. Leukocyte DNAs from 98 female patients with meningiomas were screened for the HPRT *Bam*HI polymorphism. Thirteen females (13%) were heterozygous for the HPRT/*Bam*HI polymorphism and were therefore informative for X chromosome inactivation analysis. Nine of the 13 meningiomas from these patients were analyzed. Insufficient DNA was available for the other four tumors.

Blood leukocyte DNAs from the nine patients demonstrated the pattern expected for normal polyclonal tissue (Fig. 1, *lanes a* and *b*). In *lane a,* leukocyte DNA was digested with *Bam*HI and *Pvu*II and probed with labeled HPRT700 fragment. (*Pvu*II shortens the length of the larger *Bam*HI allele from approximately 24 to 18 kilobases, thus bringing the two *Bam*HI alleles closer in size and facilitating quantitative analysis.) Hybridization to two bands of approximately 18 and 12 kilobases was observed in each case, indicating that one X chromosome contained the polymorphic *Bam*HI site, while the other did not (8, 13, 17, 18). There are nine *Hpa*II sites within the relevant region of the HPRT gene. These are heavily methylated on the active X chromosome (8). Since *Hpa*II only cleaves the unmethylated recognition sites, it will preferentially cleave the inactive HPRT allele. In the *lane b,* aliquots of leukocyte DNA were digested with *Bam*HI and *Pvu*II and then further digested with *Hpa*II. The relative intensity of both the 18- and 12-kilobase fragments decreased by approximately equal amounts. This is the expected pattern in a polyclonal tissue in which in approximately one-half of the cells the maternal X chromosome is inactive and in the other one-half the paternal X chromosome is inactive.

DNAs from the nine meningiomas were similarly analyzed (Fig. 1, *lanes c* and *d*). Digestion with *Bam*HI and *Pvu*II followed by *Hpa*II digestion resulted in selective degradation of the 18-kilobase fragment in tumors M1, M2, M4, M5, M6, and M8 and degradation of the smaller 12-kilobase fragment in the remaining three meningiomas, indicating that all nine tumors were clonal in origin. To provide further quantitative evidence of clonality, densitometry was performed and we expressed the decrease in intensities of the two alleles following *Hpa*II digestion relative to one another (19). For example, for the leukocyte DNA from patient M1, the intensity of the larger *Bam*HI allele decreased to 41% after digestion with *Hpa*II and the intensity of the smaller allele decreased to 63%. Consequently, the ratio of the greater remaining intensity percentage to the lesser remaining intensity percentage was 1.5. The polyclonal leukocyte DNA digestion patterns showed relative "cleavage ratios" near to the expected value of 1.0 (mean, 1.3) (Table 1). In contrast, the *Hpa*II cleavage ratios of the meningioma tumor DNAs ranged from 2.1 to 9.5 (mean, 4.6), which is consistent with a clonal origin of these tumors (Table 1). Although all nine tumors had ratios higher than any of the leukocyte DNAs, three of nine had ratios <3.0. This may represent contamination by nontumor cells.

These nine meningiomas were also screened for loss of heterozygosity at loci on chromosome 22 using multiple polymorphic DNA markers. At least three tumors appeared to have lost all or part of one copy of chromosome 22, while at least five retained heterozygosity for the alleles tested (Table 1). In no instance were interstitial deletions detected; however, an exhaustive study of each tumor was not performed. For tumors M1, M2, M3, M5, and M6, karyotypic analysis of cultured tumor cells was performed and was consistent with the tumor DNA analysis of chromosome 22 loci (data not shown). Thus, all nine meningiomas were monoclonal in origin irrespective of whether one or two copies of chromosome 22 were present.

Schwannomas. Among 95 female patients with schwannomas, one of whom had NF2, 11 patients (12%) were heterozygous for the HPRT *Bam*HI polymorphism. X chromosome inactivation analysis was completed on eight tumors, one of which represented a recurrence of the same tumor after 2 years. Blood leukocyte DNAs from all patients demonstrated the expected polyclonal pattern (Fig. 2, *lanes a* and *b*), namely, the relative density of both the 18- and 12-kilobase fragments decreased by about the same amount following digestion with *Hpa*II (Table 1).

Clonal analysis of the schwannomas revealed selective loss of the larger 18-kilobase allele in seven of the tumors (Fig. 2, *lanes*

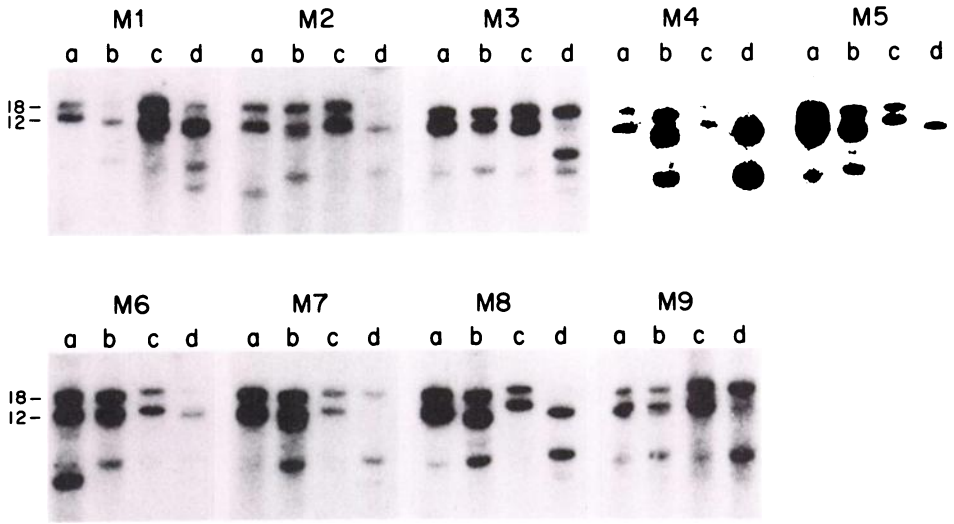

Fig. 1. Clonal analysis of meningiomas from patients 1–9 using HPRT700 as a probe. DNAs from blood leukocytes (*lanes a* and *b*) or tumors (*lanes c* and *d*) were digested with *Bam*HI and *Pvu*II. One aliquot was not digested further (*lanes a* and *c*), while the other was digested with *Hpa*II (*lanes b* and *d*). *Left ordinate,* restriction fragment size in kilobases.

Table 1 *HPRT allele cleavage ratios and loss of heterozygosity at chromosome 22 loci in meningiomas and schwannomas*

DNA from blood leukocytes or tumors was digested with appropriate restriction endonucleases and hybridized with probes as described in the text. The X chromosome cleavage ratio reflects the relative *Hpa*II sensitivity of the two HPRT alleles in the leukocytes or tumors. Tumors were also scored for loss of heterozygosity (LOH) at chromosome 22 loci.

	X chromosome cleavage ratio		Chromosome 22	
Tumor	Leukocyte	Tumor	Informative loci	LOH
Meningiomas				
M1	1.5	3.2[a]	b, c, e, g[b]	0/4[c]
M2	1.4	2.4	b, d, e, g	4/4
M3	1.5	5.7	a	0/1
M4	1.1	9.5	d, e	2/2
M5	1.4	2.1	h	0/1
M6	1.1	2.9	c, f, g	0/3
M7	1.2	4.1	ND[d]	ND
M8	1.1	8.2	d	1/1
M9	1.5	2.9	f	0/1
Schwannomas				
A1	1.3	9.8	b, f, g	0/3
A2	1.2	4.7	b, d, f	0/3
A3	1.2	2.2	ND	ND
A4	1.2	1.2	g	0/1
A5	1.3	2.1	f, g	0/2
A6	1.3	5.2	b, c	0/2
A7	1.3	3.6	b, c	0/2
A8	1.0	2.6	b	0/1

[a] Cleavage ratio indicates the relative decrease in intensities of the two HPRT alleles following *Hpa*II digestion.
[b] Informative loci include a, IGLV (IGLV/*Taq*I), b, CRYB2 (BCRY11/*Pst*I); c, D22S1 (pMS3-18/*Bgl*II); d, D22S28 (W23C/*Bgl*I); e, D22S29 (W22D/*Taq*I); f, D22S23 (W24F/*Eco*RV); g, D22S23 (W24F/*Sac*I); h, D22S24 (W21G/*Msp*I).
[c] Number of loci with allele loss/number of informative loci.
[d] ND, not done due to limited availability of DNA.

c and *d*). Four of the schwannomas, including A1, A2, A6, and A7, were clearly monoclonal, with cleavage ratios of 9,8, 4.7, 5.2, and 3.6, respectively. Tumor A2 was the schwannoma obtained from a patient with NF2. Three schwannomas (A3, A5, and A8) were probably monoclonal, with cleavage ratios of 2.2, 2.1, and 2.6, respectively, and one (A4) appeared polyclonal, with a ratio of 1.2. The ambiguous results again are likely due to an admixture of tumor cells with normal cells.

The schwannomas were also screened for loss of heterozygosity at several loci on chromosome 22 (Table 1). At least seven of the eight tumors, including tumors A6 and A7 from the same patient, retained heterozygosity for the chromosome 22 loci examined. Thus, schwannomas with a normal chromosome 22 complement were monoclonal.

DISCUSSION

Clonal analysis of two histological types of nervous system tumors has revealed that most, if not all, meningiomas and schwannomas are monoclonal in origin. It is not known whether the locus on chromosome 22 promoting meningioma formation is the same as that promoting schwannoma formation. In a prior study, 40% of meningiomas were associated with loss of chromosome 22 alleles (3). The current data agree with this. In contrast, we found that none of seven acoustic neuromas had lost chromosome 22 alleles. While the sample number is small, this finding suggests that the prior figure of approximately 40% of schwannomas showing chromosome 22 loss may be an overestimate (2).

One difficulty in the interpretation of these results was that some of the DNA digestion patterns were not clearly monoclonal but rather were skewed toward a polyclonal pattern. The most likely reason for these ambiguous patterns was contamination with blood or with normal stromal tissue. In addition, methylation patterns may be aberrant in tumor tissue causing, for example, one or both alleles to be refractory to *Hpa*II digestion. Furthermore, variability in DNA loading, electrophoretic transfer, and hybridization efficiency may cause inaccuracies. For these reasons, we place more emphasis on a positive, namely, monoclonal, result than on a negative polyclonal result. However, we cannot entirely exclude the possibility that an occasional tumor, such as A4, is truly polyclonal.

The molecular mechanisms of tumorigenesis of human meningiomas and schwannomas are not yet fully understood. Although both are often associated with loss of part or all of one copy of chromosome 22, the majority of tumors do not

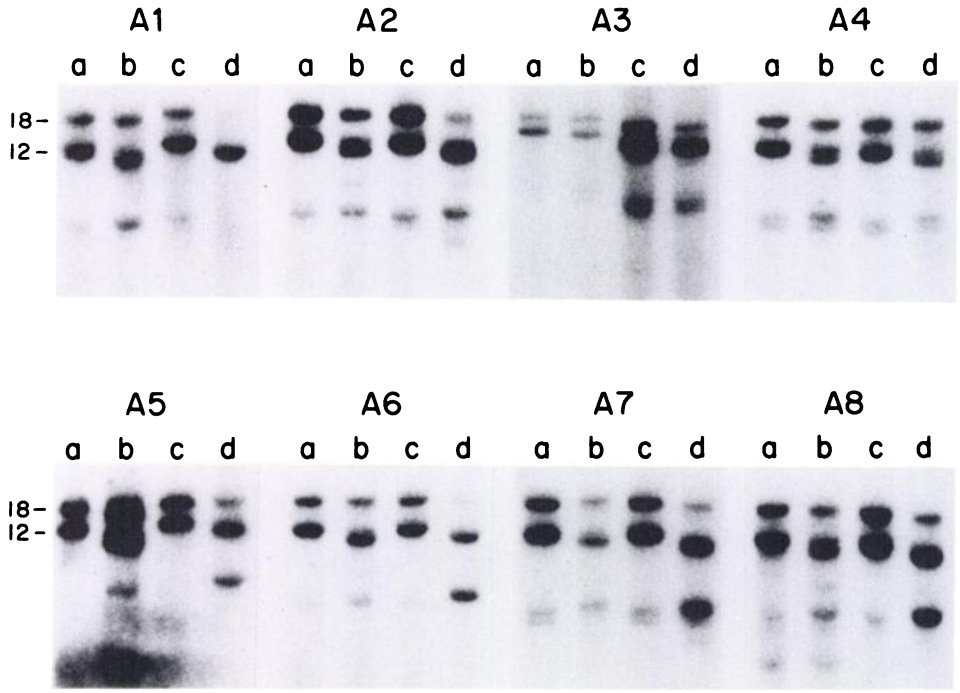

Fig. 2. Clonal analysis of schwannomas using HPRT700 as a probe. Tumors A6 and A7 were from the same patient. DNAs from blood leukocytes (*lanes a* and *b*) or tumors (*lanes c* and *d*) were digested as described in Fig. 1. *Left ordinate:* restriction fragment size in kilobases.

show such losses using currently available probes. One explanation for this is that some tumors contain a small mutation, possibly a point mutation, on chromosome 22 beyond the limits of detection by currently available probes. However, other possibilities such as a second as yet undetected tumorigenic locus have not been excluded. For acoustic neuromas, a further possibility has been suggested, namely, stimulation of Schwann cells by a growth factor. In one study, biologically active glial growth factor-like activity was demonstrated in acoustic neuromas (20). In another study, elevated levels of basic fibroblast growth factor mRNA were found (21). Thus, one possible mechanism of tumorigenesis in those tumors not showing loss of chromosome 22 loci might be the stimulation of mitosis of multiple Schwann cells by a small number of tumor-initiating cells or by an exogenous source of growth factor. If such were the case, the tumor mass would be polyclonal in origin and a genetic loss, even if present in a portion of cells, might not be detectable. However, we have demonstrated that even those tumors with no detectable loss on chromosome 22 are monoclonal in origin. This is strong evidence against a diffusible growth factor causing the tumor by stimulating multiple cells. However, it does not exclude the possibility that an autocrine growth factor system is involved at an intracellular level, nor does it differentiate between the possibilities of a small (or point) mutation on chromosome 22 *versus* the possibility of a second tumorigenic locus.

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