Common Pathogenetic Mechanism for Three Tumor Types in Bilateral Acoustic Neurofibromatosis

BERND R. SEIZINGER, GUY ROULEAU, LAURIE J. OZELIUS, ANDREW H. LANE, PETER ST GEORGE-HYSLOP, SUSAN HUSON, JAMES F. GUSELLA, ROBERT L. MARTUZA

Bilateral acoustic neurofibromatosis (BANF) is a genetic defect associated with multiple tumors of neural crest origin. Specific loss of alleles from chromosome 22 was detected with polymorphic DNA markers in two acoustic neuromas, two neurofibromas, and one meningioma from BANF patients. This indicates a common pathogenetic mechanism for all three tumor types. The two neurofibromas were among three taken from the same patient, and both showed loss of identical alleles demonstrating that the same chromosome suffered deletion in both tumors. The third neurofibroma from this patient showed no detectable loss of heterozygosity, which suggests the possibility of a more subtle mutational event that affects chromosome 22. In the two acoustic neuromas, only a portion of chromosome 22 was deleted, narrowing the possible chromosomal location of the gene that causes BANF to the region distal to the *D22S9* locus in band 22q11. The identification of progressively smaller deletions on chromosome 22 in these tumor types may well provide a means to clone and characterize the defect.

BILATERAL ACOUSTIC NEUROFIBROMATOSIS (BANF) (also known as central neurofibromatosis) is an autosomal dominant human disorder characterized by neoplasia of cells of neural crest origin (*1*). Individuals possessing this gene defect show a predisposition to a number of different tumors of the central and peripheral nervous system, including meningiomas, gliomas, and spinal neurofibromas (*1*). In particular, the hallmark of this disease is the bilateral occurrence of acoustic neuromas, which are Schwann cell–derived tumors of the vestibular branch of the eighth cranial nerve (*2*). The consequences of tumors in patients with BANF can be severe; deafness, paralysis, and other neurological morbidity and mortality can occur within the first few decades of life (*1*). The primary biochemical defect underlying tumor formation has not yet been elucidated.

The same types of tumors that are associated with BANF also occur with a relatively greater frequency singly in individuals who did not inherit the tendency (*1, 3*). In contrast to the bilateral tumors of BANF, sporadic acoustic neuromas are typically unilateral. Using polymorphic DNA markers, we have recently demonstrated that specific loss of genes on chromosome 22 is a frequent event in these sporadic cases of acoustic neuroma (*4*). We have now extended this approach to investigate tumors of several different histological types from patients with BANF. Our results indicate that, like spontaneous noninherited tumors, hereditary acoustic neuromas also display a specific loss of constitutional heterozygosity for chromosome 22. Furthermore, two other tumor types in these patients, meningioma and neurofibroma, also show loss of alleles on chromosome 22, which suggests a common pathogenetic mechanism possibly related directly to the primary defect.

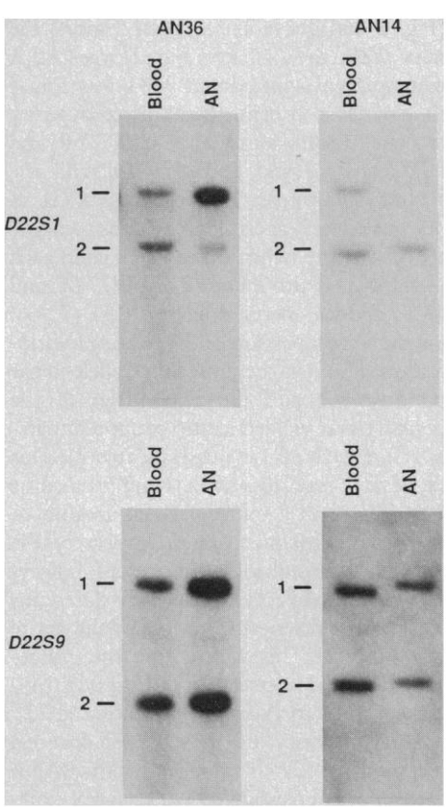

Fig. 1. Loss of constitutional heterozygosity for distinct chromosome 22 loci in acoustic neuromas from patients with BANF. DNA was isolated from tumor specimens and corresponding normal tissue (peripheral leukocytes), digested with appropriate restriction enzymes, fractionated by agarose gel electrophoresis, and transferred to nylon membrane (*4*). Southern blots were hybridized to ^{32}P-labeled DNA probes pMS3-18 (*D22S1*) and p22/34 (*D22S9*), respectively. Patient designations are shown above the autoradiograms. Numbers on the left indicate the observed alleles, with "1" and "2" referring to the larger and smaller allelic restriction fragments, respectively. pMS3-18 reveals an RFLP in Bgl II–digested human DNA with fragments of 9.5 kb ("1" allele) and 6.5 kb ("2" allele) (*8*). p22/34 detects a RFLP with fragments of 5.8 kb ("1" allele) and 3.2 kb ("2" allele) in Taq I–digested DNA (*7*). Blood, DNA from peripheral blood leukocytes; AN, DNA from acoustic neuroma tissue.

Genomic DNA was isolated from tumor tissue and lymphocytes from six patients who had confirmed BANF according to the recently established National Institutes of Health criteria (*5*). To detect the somatic loss of chromosome 22 sequences, we typed tumor and corresponding normal (leukocyte) DNA with four polymorphic DNA markers for chromosome 22: *SIS*, the platelet-derived growth factor β-chain locus (homologous to the *sis*-oncogene) mapping to 22q12.3–q13.1 (*6, 7*); *D22S1*, an anonymous DNA locus detected by probe pMS3-18 and mapping to 22q11.2–q13 (*7, 8*); *D22S9*, an anonymous DNA locus detected by probe p22/34 and mapping to 22q11 (*7*); and *IGLC*, the constant region of the λ light chain of immunoglobulin at 22q11 (*7*). Four of the six patients were heterozygous in their normal tissue for at least one of the four polymorphic DNA markers, and we were therefore able to determine whether loss of constitutional heterozygosity had occurred in their respective tumor tissue. The results of the analysis of these four patients are presented in Table. 1.

In a previous report (*4*), we determined that acoustic neuroma tissue from patient AN14 had lost heterozygosity for the *D22S1* locus. We have now reanalyzed this tumor, along with a new acoustic neuroma from patient AN36, with markers *D22S1* and *D22S9*. Patients AN36 and AN14 were constitutionally heterozygous at both *D22S1* and *D22S9*. In DNA from the corresponding acoustic neuroma tissue of each patient, heterozygosity was lost for *D22S1*, but not for *D22S9* (Fig. 1). These two DNA markers both map on the long arm of chromosome 22 with *D22S9* located closer to the centromere (*7–9*). Our findings are consistent with either a partial deletion or a

B. R. Seizinger, G. Rouleau, L. J. Ozelius, P. St George-Hyslop, J. F. Gusella, Neurogenetics Laboratory, Massachusetts General Hospital, and Department of Genetics, Harvard Medical School, Boston, MA 02114.
A. H. Lane, Neurogenetics Laboratory, Massachusetts General Hospital, and Department of Genetics, Harvard Medical School, Boston, MA 02114 and Department of Surgery, Neurosurgery Service, Massachusetts General Hospital and Harvard Medical School, Boston, MA 02114.
S. Huson, Section of Medical Genetics, University of Wales College of Medicine, Heath Park, Cardiff, United Kingdom.
R. L. Martuza, Department of Surgery, Neurosurgery Service, Massachusetts General Hospital and Harvard Medical School, Boston, MA 02114.

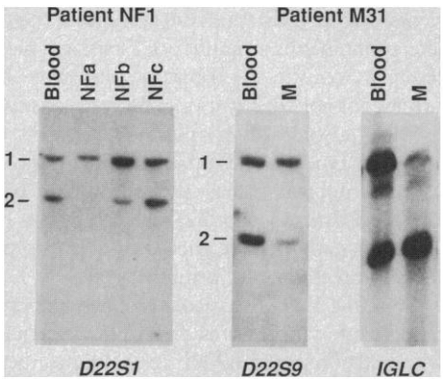

Fig. 2. Loss of constitutional heterozygosity for chromosome 22 loci in neurofibroma and meningioma tumors from BANF patients. DNA from tumor specimens and corresponding peripheral leukocytes was digested with appropriate restriction enzymes (Table 1) and analyzed by the Southern blot technique (*4*). Filters were hybridized with radiolabeled probes pMS3-18 (*D22S1*), p22/34 (*D22S9*), and HuλC2 (*IGLC*), respectively. Patient designations are shown above the autoradiograms. Numbers on the left indicate the observed alleles with "1" and "2" referring to the larger and smaller allelic restriction fragments, respectively. The RFLPs detected by probes pMS3-18 and p22/34 are described in the legend to Fig. 1. HuλC2 reveals a multi-allele RFLP represented by fragments of 8, 13, 18, and 23 kb in Eco RI–digested genomic DNA (*7*). Blood, DNA from peripheral blood leukocytes; NF, DNA from neurofibroma tissue; M, DNA from meningioma tissue.

Table 1. Loss of heterozygosity on chromosome 22 in different tumor types from patients with bilateral specimens and corresponding normal tissue (peripheral leukocytes) as described (*4*). Approximately 5 μg electrophoresis, transferred to nylon membranes, and hybridized to ^{32}P-labeled probe DNA (*4*). Probes length polymorphisms (RFLPs) in human genomic DNA with the indicated restriction enzymes: (nerve growth factor–β *NGFB*) (*13*); G8 (*D4S10*) (*14*); Dry 5-1 (*D10S1*) (*7*); pEJ6.6 (*HRAS1*) (*15*); (*D13S5*) (*18*); pAW101 (*D14S1*) (*19*); B74 (*D18S3*) (*7*); pC3 (*C3*) (*20*); and pGSH8 (*D21S17*) (*21*). heterozygosity: "12" indicates heterozygosity (even though different pairs of alleles may be present for and loss of the smaller allelic fragment relative to normal tissue DNA; and "2" indicates continued was tested but was uninformative because it did not display heterozygosity a dash has been entered to of limited availability of DNA.

Patient*	Marker†								
	SIS (H;22)	*D22S1* (B;22)	*D22S9* (T;22)	*IGLC* (E;22)	*NGFB* (B,1)	*NGFB* (T;1)	*D4S10* (H;4)	*D10S1* (T;10)	*HRAS1* (T;11)
AN36	—	1	12	—	12	—	12		—
AN14	—	2	12	—	—	—	—	12	—
NF1a	—	1	—	—	12	12	12	—	12
NF1b	—	1	—	—	12	12	12	—	12
NF1c	—	12	—	—	12	12	12	—	12
M31	—	—	1	2	—	—	12	—	12

*Tissues used were as follows: (AN36) acoustic neuroma; (AN14) acoustic neuroma; (NF1a) neurofibroma a, cervical neath the markers are the single-letter abbreviations for the restriction enzymes used (H, Hind III; B, Bgl II; T, Taq I; *D22S9* and *IGLC* are at 22q11).

mitotic recombination on 22q with a breakpoint between 22q11 *D22S9* and 22q11.2–q13 (*D22S1*).

To determine whether the loss of heterozygosity was the result of a reduction to hemizygosity, we analyzed the hybridization signals from Fig. 1 by densitometry, relative to signals obtained when the same filters were rehybridized with probes for control loci on other chromosomes (see Table 2). As shown in Table 2, the ratio of the normalized hybridization signals for the *D22S1* marker in acoustic neuroma DNA versus DNA from the corresponding normal tissue was approximately 1:2 in both cases. This indicates deletion of one copy of the *D22S1* locus in each of these tumors rather than a mitotic recombination or gene conversion event. The DNA from the tumors displayed no change in the normalized hybridization signal for the *D22S9* marker relative to DNA from normal tissue (Table 2). These data provide further support for the conclusion that a partial deletion occurred in each case with a breakpoint distal to the *D22S9* locus in band 22q11.

In the densitometric analysis, the hybridization signals corresponding to the deleted allele of the *D22S1* locus were diminished by 83 and 78% in the tumors from patients AN36 and AN14, respectively. The remaining hybridization signals might be caused by contaminating nontumor cells (vascular or connective tissue), which may be present in primary surgical specimens (*4, 10*). Alternatively, the tumors might consist of a mosaic of cells with normal karyotype and cells with deletions on chromosome 22, which has been observed, for example, in cytogenetic studies of cultured meningiomas (*11*).

In addition, we obtained three neurofibromas from the upper cervical spinal region of patient NF1. Of the four DNA markers typed, the patient's normal DNA displayed heterozygosity only for *D22S1*. Constitutional heterozygosity for *D22S1* was lost in two of the three neurofibromas (Fig. 2 and Table 1). In both tumors, the same allele (the 2 allele in Fig. 2) was lost. A meningioma from patient M31 was found to have lost constitutional heterozygosity for the chromosome 22 loci *D22S9* and *IGLC* (Fig. 2 and Table 1). As with the acoustic neuromas, marked reductions in one allele were determined by densitometric analysis of the hybridization signals in each of the two neurofibromas of NF1 (85 and 53%) and the meningioma of M31 (72%). Similarly, comparison of the relative hybridization signal for the nondeleted allele in the tumor tissue and the two alleles in the normal tissue yielded a ratio of approximately 1:2 in each of these cases (Table 2). This suggests a "true" loss of loci on chromosome 22 rather than a mitotic recombination or loss with subsequent reduplication of the remaining chromosome. At present, however, we are unable to conclusively differentiate a partial deletion from loss of the entire chromosome. This will require the characterization and application of additional localized DNA markers for chromosome 22.

The specificity of the loss of loci on chromosome 22 in tumors from BANF patients was investigated with several polymorphic DNA markers for ten other chromosomes (Table 1). In every case where constitutional heterozygosity was observed, heterozygosity was also maintained in the corresponding tumor tissue. Thus, these tumors exhibited a remarkable genome stability with chromosomal losses being highly specific to chromosome 22. The data in Table 1 also provide assurance that the differences between tumor and normal DNA observed for chromosome 22 did not arise from simply mispairing of the samples or unreliability of the applied techniques, since filters first used for chromosome 22 studies then were rehybridized with the probes for other chromosomes.

Our previous report on sporadic acoustic neuromas included two tumors from BANF patients that maintained constitutional heterozygosity for either *D22S1* or *SIS* (*4*). Although these tumors, like the third neurofibroma from patient NF1 in the current study, showed no apparent loss of heterozygosity on chromosome 22, they might well have undergone mitotic or mutational events on this autosome that could not be detected with the available genetic markers. The possibilities would include the occurrence of point mutations in a specific gene, or small interstitial deletions, as well as larger deletions or mitotic recombination events with breakpoints distal to the informative loci. Additional high-quality markers from many different regions on chromosome 22 would facilitate the elucidation of such cases.

This report demonstrates that specific loss of genes on chromosome 22 is a frequent event not only in sporadic cases of acoustic neuroma (*4*) but also in the heritable form in BANF. It is likely that both types of acoustic neuroma result from similar mechanisms involving the same gene locus. Moreover,

acoustic neurofibromatosis. High relative molecular mass DNA was isolated from surgical tumor of DNA was digested to completion with appropriate restriction enzymes, fractionated by agarose gel for loci on several different chromosomes were chosen that were known to reveal restriction fragment Psis.1 (Oncor) (*SIS*) (*6*); pMS3-18 (*D22S1*) (*8*); p22/34 (*D22S9*) (*7*); HuλC2 (*IGLC*) (*7*); N8C6 pHINS321 (*INS*) (*16*); p640 (*KRAS2*) (*15*); p7F12 (*D13S1*) (*17*); pIE8 (*D13S4*) (*17*); pHUB8 The phenotype observed in the tumor tissue is shown for every case where the blood DNA displayed certain multi-allele markers); "1" indicates continued presence of the larger allele restriction fragment presence of the smaller allelic restriction fragment and loss of the larger fragment. Where the normal DNA simplify consideration of the data. The absence of an entry indicates that a marker was not tested because

Marker†									
INS (S;11)	*KRAS2* (T;12)	*D13S1* (M;13)	*D13S4* (M;13)	*D13S5* (E;13)	*D14S1* (E;14)	*D14S1* (H;14)	*D18S3* (M;18)	*C3* (S;19)	*D21S17* (B;21)
	12			12	—	12			—
	12		—	12	12	—	—	12	12
12	—	12	—	—	12	—	—	12	12
12	—	12	—	—	12	—	—	12	12
12	—	12	—	—	12	—	—	12	12
—	12	—	12		12	12	12	—	12

1; (NF1b) neurofibroma b, foramen magnum; (NF1c) neurofibroma c, cervical 2; (M31) meningioma. †Under- E, Eco RI, S, Sac I; M, Msp I) and the chromosome location (*SIS* is at 22q12.3–13.1; *D22S1* is at 22q11.2–q13; and

Table 2. Quantitative densitometry of probe hybridization for tumors from patients with bilateral acoustic neurofibromatosis. Southern blots that had been hybridized to probes for chromosome 22 (Fig. 1) were freed of these probes in distilled water for 2 hours at 65°C and rehybridized with probes for polymorphic loci on other chromosomes ("control chromosomes"). The autoradiograms from all hybridizations were analyzed by scanning densitometry in an LKB Ultrascan XL; peak areas corresponded to each hybridization signal as quantitated by electronic integration. To determine whether any chromosome 22 alleles had been duplicated in the tumors, we normalized hybridization signals specific for chromosome 22 relative to hybridization signals for control chromosome probes in the same sample, and then calculated a ratio of the normalized values for each tumor/normal tissue pair. In order to validate our approach of normalizing the hybridization signals for chromosome 22 probes, we previously compared hybridization signals for pairs of markers from other chromosomes. These invariably gave constant ratios for tumor and normal DNA (*4*). Chrom., chromosome; NGFB, nerve growth factor–β.

Patient	Tissue	Chrom. 22 locus	Control locus	Chrom. 22/ control chrom.	Tumor/ normal
AN36	Acoustic neuroma	*D22S1*	*D21S17*	0.77	0.48
	Leukocytes			1.62	
AN36	Acoustic neuroma	*D22S9*	*NGFB*	0.70	0.99
	Leukocytes			0.71	
AN14	Acoustic neuroma	*D22S1*	*D21S17*	0.09	0.51
	Leukocytes			0.17	
AN14	Acoustic neuroma	*D22S9*	*NGFB*	0.94	1.05
	Leukocytes			0.90	
NF1a	Neurofibroma a	*D22S1*	*D21S17*	2.23	0.68
	Leukocytes			3.26	
NF1b	Neurofibroma b	*D22S1*	*D21S17*	1.78	0.55
	Leukocytes			3.26	
NF1c	Neurofibroma c	*D22S1*	*D21S17*	3.28	1.01
	Leukocytes			3.26	
M31	Meningioma	*D22S1*	*NGFB*	1.01	0.46
	Leukocytes			2.21	

this specific chromosomal alteration apparently represents a common pathogenetic mechanism for different histologic tumor types in BANF.

By analogy to the pathogenetic mechanism proposed for retinoblastoma and Wilms' tumor (*12*), the loss of alleles on chromosome 22 may serve to unmask a recessive mutant allele by elimination of the homologous wild-type gene. This model predicts that the inherited defect in BANF is in a gene on chromosome 22. Loss of the same, presumably wild-type, copy of chromosome 22 in two separate tumors of BANF patient NF1 is consistent with this hypothesis. Alternatively, the BANF gene may be located on another autosome; loss of genes on chromosome 22 may then represent a secondary event promoting tumorigenesis in certain cell types. The detection of familial cases of acoustic neuroma exhibiting only partial deletion of chromosome 22 narrows the region that may contain the BANF gene, or another locus involved in the development of BANF-associated tumors, to the long arm of chromosome 22 (distal to *D22S9*). The identification of additional tumors with progressively smaller deletions represents a potential strategy for cloning of this locus. Further characterization of the defect might not only have a profound impact on diagnosis and treatment of these tumors in both their sporadic and familial forms but could also provide a greater understanding of fundamental control mechanisms operating in the normal developing nervous system.

REFERENCES AND NOTES

1. R. Eldridge, *Adv. Neurol.* **29**, 57 (1981); R. L. Martuza and R. G. Ojemann, *Neurosurgery* **10**, 1 (1982); W. R. Kanter *et al.*, *Neurology* **30**, 851 (1980); S. M. Huson and D. C. Thrush, *Q. J. Med.* **218**, 213 (1985).
2. L. J. Rubenstein, *Tumors of the Central Nervous System* (Armed Forces Institute of Pathology, Washington, DC, 1972).
3. B. S. Schoenberg, B. W. Christine, J. P. Whisnant, *Am. J. Epidemiol.* **104**, 499 (1976).
4. B. R. Seizinger, R. L. Martuza, J. F. Gusella, *Nature (London)* **322**, 664 (1986).
5. J. Mulvihill, Ed., *Neurofibromatosis Res. Newsl.* **2**, No. 7 (1986).
6. D. Barker and R. White, *Cytogenet. Cell Genet.* **37**, 250 (1984); C. Julier *et al.*, *ibid.* **40**, 664 (1985).
7. H. F. Willard, M. H. Skolnick, P. L. Pearson, J. L. Mandel, *ibid.* **40**(HGM8), 360 (1985).
8. D. Barker, M. Schafer, R. White, *Cell* **36**, 131 (1984).
9. C. Julier *et al.*, *Cytogenet. Cell Genet.* **40**, 665 (1985).
10. E. R. Fearon *et al.*, *Nature (London)* **318**, 377 (1985).
11. K. D. Zang, *Cancer Genet. Cytogenet.* **6**, 249 (1982).
12. W. K. Cavenee *et al.*, *Nature (London)* **305**, 779 (1983); T. P. Dryja *et al.*, *N. Engl. J. Med.* **310**, 550 (1984); W. F. Benedict *et al.*, *Science* **219**, 973 (1983); A. Koufos *et al.*, *Nature (London)* **309**, 170 (1984); S. H. Orkin, D. S. Goldman, S. E. Sallan, *ibid.*, p. 172; A. E. Reeve *et al.*, *ibid.*, p. 174; E. R. Fearon, B. Vogelstein, A. P. Feinberg, *ibid.*, p. 176.
13. X. O. Breakefield *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 4213 (1984); J. K. Darby *et al.*, *Am. J. Hum. Genet.* **37**, 52 (1985).
14. J. F. Gusella *et al.*, *Nature (London)* **306**, 234 (1983); J. F. Gusella *et al.*, *ibid.* **318**, 75 (1985).
15. D. Barker *et al.*, *Mol. Biol. Med.* **1**, 199 (1983).
16. G. I. Bell, S. Horita, J. H. Karam, *Diabetes* **33**, 176 (1984).
17. W. Cavenee *et al.*, *Am. J. Hum. Genet.* **36**, 10 (1984).
18. T. Dryja *et al.*, *Hum. Genet.* **65**, 320 (1984).
19. A. R. Wyman and R. L. White, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 6754 (1980).
20. K. Davies *et al.*, *J. Med. Genet.* **20**, 259 (1983).
21. G. D. Stewart *et al.*, *Nucleic Acids Res.* **13**, 4125 (1985).

22. We thank N. Zervas, R. Ojemann, R. Weeks, D. Shaw, and the Massachusetts General Hospital neurosurgical staff for their assistance in providing specimens, S. de la Monte for assistance in pathological interpretation, and K. Dashner and K. Riley for providing technical assistance. We are grateful to the following scientists for providing DNA probes: A. Ullrich, T. Dryja, G. Bell, C. Shih, R. Weinberg, R. White, B. White, J. L. Mandel, G. Fey, G. Stewart, and P. Leder. B.R.S. is supported by a fellowship and grant from the National Neurofibromatosis Foundation. G.R. is supported in part by the Fonds de Recherche en Santé du Quebec and G.R. and P. StG.-H. are fellows of the Medical Research Council of Canada. R.L.M. is recipient of a Teacher-Investigator Career Development Award from the National Institute of Neurological and Communicative Disorders and Stroke (NS00654). J.F.G. is a Searle Scholar of the Chicago Community Trust. Funds for this research were provided by NIH grants NS22224 and NS20025, the McKnight Foundation, the Dystonia Medical Research Foundation, and the Julieanne Dorn Fund for Neurological Research.

24 October 1986; accepted 28 January 1987