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Flanking Markers Bracket the Neurofibromatosis Type 2 (NF2) Gene on Chromosome 22

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Summary

Neurofibromatosis 2 or bilateral acoustic neurofibromatosis (NF2) is a severe autosomal dominant disorder characterized by the development of multiple tumors of the nervous system, including meningiomas, gliomas, neurofibromas, ependymomas, and particularly acoustic neuromas. Polymorphic DNA markers have revealed frequent loss of one copy of chromosome 22 in the tumor types associated with NF2. Family studies have demonstrated that the primary defect in NF2 is linked to DNA markers on chromosome 22, suggesting that it involves inactivation of a tumor suppressor gene. We have employed a combination of multipoint linkage analysis and examination of deletions in primary tumor specimens to precisely map the *NF2* locus between flanking polymorphic DNA markers on chromosome 22. The 13-cM region bracketed by these markers corresponds to 13% of the genetic length of the long arm of chromosome 22 and is expected to contain less than 5×10^6 bp of DNA. The delineation of flanking markers for *NF2* should permit accurate presymptomatic and prenatal diagnosis for the disorder and greatly facilitate efforts to isolate the defective gene on the basis of its location.

Introduction

Neurofibromatosis, one of the most common Mendelian disorders affecting the human nervous system, has two clinically distinct forms: von Recklinghausen neurofibromatosis (NF1) (Riccardi 1981) and bilateral acoustic neurofibromatosis (NF2) (Martuza and Eldridge 1988; Wertelecki et al. 1988). Both of these autosomal dominant disorders involve frequent tumors of the nervous system. NF1 is associated with benign astrocytomas of the optic-hypothalamic region and with Schwann cell tumors (neurofibromas) confined primarily to the skin, peripheral nerves, and spinal nerve roots. Schwann cell tumors of the eighth cranial nerve (acoustic neuromas) are rare. By contrast, NF2 predisposes to acoustic neuromas, the hallmark of the disorder, as well as to meningiomas, ependymomas, spinal neurofibromas, and gliomas. Central nervous system tumors of NF2 lead to deafness, vertigo, and paralysis, often requiring repeated surgical intervention. Although NF1 is more common (incidence 1/3,000), the neurological morbidity of NF2 (1/100,000) can be much more devastating.

The genetic defect causing NF2 has been mapped to chromosome 22 by family studies linking it to the DNA marker *D22S1* (Rouleau et al. 1987). The rationale for searching on chromosome 22 came from molecular genetic studies of the tumor types commonly associated with NF2 (Seizinger et al. 1987*b*). First, loss of DNA marker alleles for this autosome was detected with high frequency in sporadic cases of meningioma and acoustic neuroma (Seizinger et al. 1986, 1987*b*; Dumanski et al. 1987; Okazaki et al. 1988). Subsequently, similar losses were documented in acoustic neuromas, meningiomas, and neurofibromas obtained from patients with NF2 (Seizinger et al. 1987*b*). Taken together, these data strongly suggest that the *NF2* locus

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encodes a recessive anti-oncogene or tumor suppressor gene analogous to *RB1,* the locus involved in retinoblastoma (Cavenee et al. 1983). This view is supported by the loss of the same chromosome 22 allele in multiple independent tumors from the same NF2 patient. To precisely define the location of the *NF2* gene and thereby lay the foundation for isolating this locus, we have used a combination of linkage studies and deletion mapping in NF2-associated tumors. We have determined that the candidate genes *PDGFB,* which encodes the beta chain of the platelet-derived growth factor, and *BCR,* a gene involved in the Philadelphia chromosome translocation, are not responsible for NF2. However, the analysis permitted identification of flanking markers which bracket the defect within a 13-cM region of the long arm of the chromosome.

Material and Methods

Clinical Evaluation

After informed consent was obtained, all patients were evaluated by physical examination, brain-stem auditory-evoked response testing, and computerized tomography or magnetic resonance imaging when indicated. The diagnosis of NF2 was determined as described by Rouleau et al. (1987).

Typing of DNA Markers

High-molecular-weight DNA was isolated from blood or transformed lymphocytes as described by Rouleau et al. (1987) and from primary tumors from NF2 patients as described by Seizinger et al. (1987*b*). Approximately 5 μg DNA was digested to completion with appropriate restriction enzymes, fractionated by agarose gel electrophoresis, transferred to nylon membrane, and hybridized to ^{32}P-labeled probe DNA (Rouleau et al. 1987). The following probes, known to reveal RFLPs in human genomic DNA for loci on chromosome 22, were used: pMS3-18 (*D22S1*) (Barker et al. 1984), Psis1 (Oncor) (*PDGFB*) (Julier et al. 1988). BCR1 (Oncogene Science, Inc.) and bcr Pr-1 (*BCR*) (Groffen et al. 1984; Roschmann et al. 1987), W23C (*D22S28*) (Rouleau et al. 1989), and 22C1-18 (*D22S10*) (Hofker et al. 1985).

Linkage Calculations

Pedigree and clinical data were compiled from a single large NF2 kindred as described by Wertelecki et al. (1988). Data were analyzed using the computer programs LIPED (version 3) (Ott 1976) for two-point analysis and LINKAGE (version 4.7) (Lathrop et al. 1984) for multipoint analysis. The data were analyzed using the computer program LIPED and assuming penetrance of .95 for *NF2,* with an age-at-onset correction based on a mean of 20 years (Hodge et al. 1979) as described elsewhere (Rouleau et al. 1987). Lod scores (z) for linkage of the marker to the defect were calculated for various recombination fractions (θ).

Results

Examination of Multiple Tumors from a Single NF2 Patient

The tumor suppressor gene model of NF2 predicts that an inactivating mutation at the disease locus results in tumor formation only when the normal homologue is also inactivated by a somatic event such as deletion (Hansen and Cavenee 1986). To test this hypothesis, we have typed five independent tumors (four neurofibromas and one acoustic neuroma) from an NF2 patient heterozygous for *D22S28.* The results, shown in figure 1, indicate a reduction in the intensity of the fragment corresponding to allele 1 of the marker in all five tumors, suggesting that the somatic event in each tumor involved loss of the chromosome 22 containing this allele. The remaining signal at this position indicates the presence of a small amount of contaminating

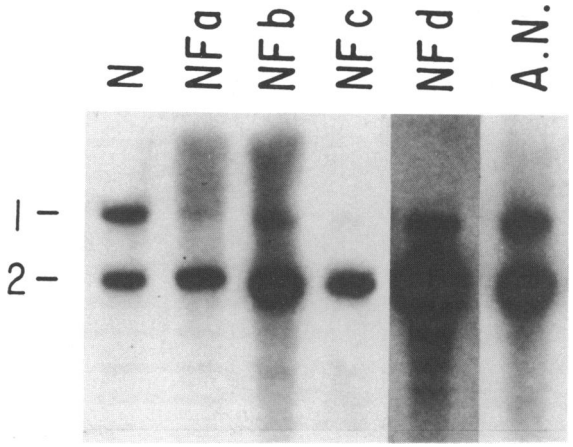

Figure 1 Loss of constitutional heterozygosity at *D22S28* in four neurofibromas (NF a–NF d) and one acoustic neuroma (A.N.) from a patient with NF2. N denotes normal leukocyte DNA tissue. The larger and smaller allelic restriction fragments seen in a *BglI* digest are denoted by 1 and 2, respectively. A relative reduction in hybridization signal for allelic fragment 1 is observed in all five tumors from this patient, suggesting that the inherited *NF2* defect resides on the chromosome 22 homologue containing allele 2.

normal tissue in the tumor specimen. Allele 2 of the *D22S28* locus therefore marks the chromosome carrying the defective *NF2* allele, although the absence of affected relatives made it impossible to confirm this by family studies.

Candidate Markers

The breakpoint cluster region, *BCR*, has been implicated in neoplasia, since it marks the chromosome 22 breakpoint of the t(9;22) Philadelphia chromosome characteristically seen in chronic myelogenous leukemia (CML) (Groffen et al. 1984; Roschmann et al. 1987). To determine whether a mutation at the *BCR* locus underlies NF2, an RFLP was required to be informative in the very large NF2 pedigree previously used to establish that the disorder was linked to *D22S1* (Rouleau et al. 1987; Wertelecki et al. 1988). Consequently, we screened the probe BCR1 for RFLPs in this kindred and discovered a single site *Bst*EII polymorphism, with allelic fragments of 9.0 kb and 6.0 kb with a frequency of 66% and 34%, respectively. We also detected a single site *Pvu*II RFLP with allelic fragments of 4.9 kb and 2.6 kb with a frequency of 96% and 4%, respectively. These polymorphisms are inherited in a Mendelian fashion and are not in linkage disequilibrium with each other or with the *Taq*I polymorphism described elsewhere for the 5′ BCR probe (Roschmann et al. 1987).

Members of the large NF2 kindred were typed for all three polymorphisms displayed by the BCR probes. The lod score was $-\infty$ at $\theta = 0$ (table 1), indicating that recombination occurred between *BCR* and the genetic defect. Thus, the locus involved in formation of the Philadelphia chromosome of CML is not the site of the defective gene in NF2.

PDGFB (the homologue of the *SIS* oncogene), which encodes the platelet-derived growth factor β chain, has been reported to be rearranged in a family with a t(14;22) chromosome associated with inherited meningiomas (Bolger et al. 1985), a tumor type which also occurs with high frequency in NF2. This marker displays a frequent *Hin*dIII polymorphism (Julier et al. 1988), permitting its linkage relationship with *NF2* to be assessed as shown in table 1. As with *BCR*, the detection of recombination with *NF2* excluded *PDGFB* as the site of the primary defect in this disorder.

Demonstration of Flanking Markers

To precisely map the *NF2* locus on the long arm of chromosome 22, we have used a chromosome 22 phage library to generate a number of polymorphic DNA markers and have ordered these into a genetic map by using the Venezuela reference pedigree (Tanzi et al. 1988). The markers of interest with respect to *NF2* are those surrounding *D22S1*, the most closely linked marker to the disorder (peak lod score $[\hat{z}] = 3.35$, $\hat{\theta} = 0$). *BCR* is located 12 cM closer to the centromere than is *D22S1*, while *D22S28* and *PDGFB* are 13 cM and 24 cM distal to *D22S1*, respectively.

To place *NF2* within the overall chromosome 22 linkage map, we performed multipoint linkage analysis using the LINKMAP program (Lathrop et al. 1984) to vary the position of *NF2* relative to fixed positions of *BCR*, *D22S1*, *D22S28*, and *PDGFB*. The results are shown in figure 2. Obligate recombination of the disease gene with the markers *BCR*, *D22S28*, and *PDGFB* explains the likelihood curve plunges to $-\infty$. The maximum likelihood estimate of the location of the *NF2* gene places it between *BCR* and *D22S28*. This location is favored by odds of 23:1 over a position proximal to *BCR* and by more than 3×10^4:1 over any location distal to *D22S28*. Because no recombinants were detected with *D22S1*, multipoint analysis could not resolve whether the disease gene is proximal or distal to

Table 1

Lod Scores for Linkage of *NF2* to Loci on Chromosome 22

	θ							
Marker Locus	.00	.01	.05	.10	.20	.30	.40	$\hat{z}$, $\hat{\theta}$
BCR	$-\infty$	.81	1.50	1.66	1.44	.97	.41	1.66, .11
D22S1	3.35	3.30	3.11	2.83	2.18	1.44	.67	3.35, .00
D22S28	$-\infty$	−2.31	−.14	.65	.99	.75	.34	1.00, .19
PDGFB	$-\infty$	.27	.97	1.16	1.07	.77	.38	1.17, .13

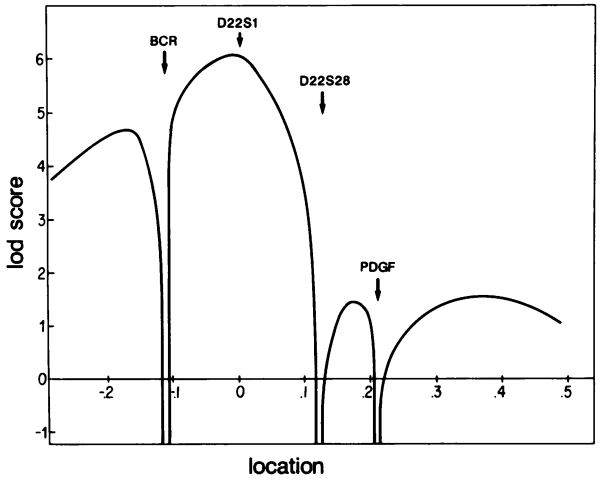

Figure 2 Multipoint linkage analysis of *NF2, BCR, D22S1, D22S28,* and *PDGFB.* The computer program LINKAGE was used to calculate lod scores for *NF2* at various locations relative to the known positions of the four DNA loci. Multiple peaks are observed with a lod score of 6.0 at *D22S1,* 4.6 proximal to *BCR,* and 1.4 between *D22S28* and *PDGFB.* The curve plunges to $-\infty$ at *BCR, D22S28,* and *PDGFB* because of obligate recombination events between the disease locus and each of these marker loci.

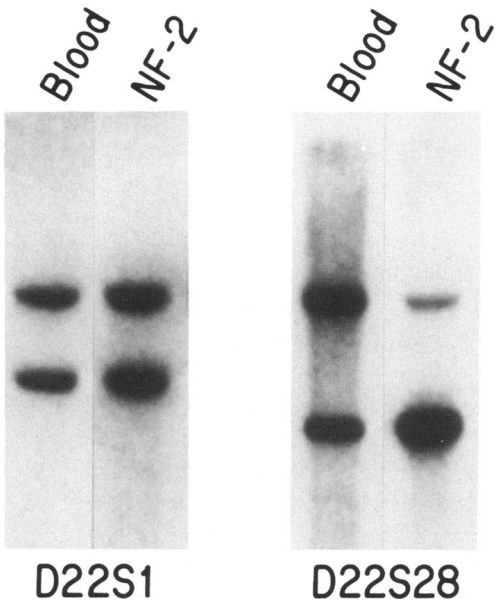

Figure 3 Loss of constitutional heterozygosity at *D22S28* but not at *D22S1* in a neurofibroma (lane NF-2) from a patient with NF2. Both blood and tumor DNA show roughly equal signal for both allelic *Bgl*II fragments at *D22S1.* However, at *D22S28,* signal for the larger allelic *Bgl*I fragment is dramatically reduced relative to that for the smaller allelic fragment, despite the tumor lane being more heavily loaded with DNA than is the blood DNA lane. The remaining signal for the larger allelic fragment at *D22S28* is probably due to a small amount of normal tissue contaminating the tumor specimen. The data suggest that the tumor DNA reflects a deletion with a breakpoint between *D22S1* and *D22S28.*

this marker. However, *NF2* is clearly flanked by *BCR* and *D22S28,* in a region of 25 cM, or one-fourth of the genetic map of 22q.

Limiting the Location of NF2 by Tumor Studies

Demonstration of loss of alleles in acoustic neuromas from patients with NF2 has implicated the involvement of a tumor suppressor gene in this disorder (Seizinger et al. 1987*b*). We have identified several tumors from NF2 patients that show only partial loss of chromosome 22, suggesting the possibility of further localizing the disease gene (Seizinger et al. 1987*b*). Recently, we have analyzed a neurofibroma from an NF2 patient who is constitutionally heterozygous—and therefore informative—for RFLPs at both *D22S1* and *D22S28.* In the tumor DNA from this patient, *D22S1* remained heterozygous for the frequent *Bgl*II polymorphism. However, for *D22S28,* only one allele was present in the tumor DNA, as demonstrated by reduction to hemizygosity of the *Bgl*I RFLP for this marker (fig. 3). Most markers distal to *D22S28* were uninformative in this tumor—with the exception of *D22S21,* which is located at the terminus of the 22q genetic map (Rouleau et al. 1989). Maintenance of heterozygosity for *D22S21* suggests that this tumor has suffered an interstitial deletion on chromosome 22, with the proximal breakpoint between *D22S1* and *D22S28.* Thus, the *NF2* gene is likely to be at or distal to this breakpoint, and, combined with the linkage mapping data, this result indicates that *NF2* is flanked by *D22S1* and *D22S28.*

Discussion

NF2 is a serious disease which is often not detected until symptoms appear. A predictive test would significantly modify patient management, prognosis, and family planning. At-risk individuals found to carry the gene could be monitored for the earliest signs of tumor formation, potentially permitting surgical intervention before the development of hearing loss or other permanent neurologic dysfunction. The identification of flanking markers should permit accurate preclinical diagnosis for the family presented here. However, more widespread use of these loci for presymptomatic or prenatal diagnosis should await the evaluation of additional NF2 families in order to rule out nonallelic genetic heterogeneity. Testing additional families will also lead to more

accurate estimates for recombination between *NF2* and the linked markers.

The precise localization the *NF2* gene on the genetic linkage map of chromosome 22 will also allow its role in the development of meningiomas to be more carefully examined. Meningiomas are one of the most frequent sporadic tumors of the central nervous system and have been associated with loss of heterozygosity for markers on chromosome 22 (Dumanski et al. 1987; Seizinger et al. 1987*a;* Okazaki et al. 1988). These tumors are also observed frequently in NF2 patients, suggesting that the critical genetic region involved in development of meningioma might be coincident with the *NF2* gene. Partial loss of chromosome 22 has been observed in meningiomas and revealed that, like *NF2,* the meningioma locus is telomeric to *D22S1.* However, additional evidence suggests that it might be even more distal than the *NF2* gene (Bolger et al. 1985; Dumanski et al. 1987; Okazaki et al. 1988). Continued deletion mapping in both types of tumors by using markers that have been genetically mapped with precision should permit definitive resolution of whether a defect at a single genetic locus is common to both types of brain tumors.

A recent report showing that chromosome 22 is the most frequently lost chromosome in colonic tumors from patients with hereditary polyposis coli (Okamoto et al. 1988) suggests that the recessive anti-oncogene responsible for NF2 may also play an important role in other human tumors outside the nervous system. Monitoring for loss of alleles from chromosome 22, particularly for the markers closest to *NF2,* in many tumor types might better define the actual extent of involvement of this locus in human cancer.

The area bracketed by the *NF2* flanking markers—*D22S1* and *D22S28*—comprises 13 cM, or about 13% of the minimum genetic length of the long arm of chromosome 22. The physical distance between these markers has not yet been determined, but the entire chromosome comprises some 52×10^6 bp of DNA (Kaplan et al. 1987), and consequently the flanking markers for *NF2* are unlikely to be separated by more than about 5×10^6 bp. Although this constitutes a large DNA segment, recent technical advances, including pulsed-field gel electrophoresis (Schwartz and Cantor 1984) and yeast artificial chromosome cloning (Burke et al. 1987), have allowed manipulation of DNA of this size. The identification of flanking markers will allow physical mapping and cloning strategies to be targeted to this relatively small portion of the chromosome. Thus, the precise mapping of *NF2* between *D22S1* and *D22S28* constitutes an important step toward isolation and characterization of the *NF2* gene on the basis of its chromosomal location.

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