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The Neurofibroma in von Recklinghausen Neurofibromatosis Has a Unicellular Origin

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Summary

von Recklinghausen neurofibromatosis (NF1) is the most common hereditary syndrome predisposing to neoplasia. NF1 is an autosomal dominant disease caused by a single gene which maps to chromosome 17q11.2. The most common symptomatic manifestation of NF1 is the benign neurofibroma. Our previous studies of tumors in NF1, studies which detected a loss of heterozygosity for DNA markers from the NF1 region of chromosome 17 in malignant tumors, did not detect a loss in neurofibromas. We report here that a more extensive study, including the analysis of neurofibromas from 19 unrelated NF1 patients by using seven probes, failed to detect a single instance of loss of heterozygosity. This finding suggests that neurofibromas are either polyclonal or monoclonal in origin but arise by a mechanism different from that of NF1 malignancies. In order to investigate the first possibility, we analyzed neurofibromas from female NF1 patients by using an X chromosome–specific probe, from the phosphoglycerokinase (PGK) gene, which detects an RFLP. The detected alleles carry additional recognition sites for the methylation-sensitive enzyme *Hpa*II, so that the allele derived from the active X chromosome is digested by *Hpa*II while the one from the hypermethylated, inactive X chromosome is not. We analyzed neurofibromas from 30 unrelated females with NF1. Eight patients were heterozygous for the PGK RFLP. By this assay, neurofibromas from all eight appeared monoclonal in origin. These results suggest that benign neurofibromas in NF1 arise by a mechanism that is different from that of malignant tumors. This mechanism may involve (*a*) an NF1 gene mutation that, by our analysis, is not detectable as a loss of heterozygosity or (*b*) a gene or genes other than the NF1 gene.

Introduction

Von Recklinghausen neurofibromatosis (neurofibromatosis type 1, or NF1) is the most common hereditary syndrome predisposing to neoplasia, affecting approximately 1/3,000 live births in the United States (Crowe et al. 1956). The pleiotropic manifestations of this disease include cutaneous and subcutaneous neurofibromas, café au lait spots, Lisch nodules, learning disabilities, skeletal abnormalities, and malignancies of the central and peripheral nervous system (Riccardi and Eichner 1986). The most common tumor occurring in NF1 is the neurofibroma, a tumor composed primarily of Schwann cells and fibroblasts with numerous perineural, endothelial, and mast cells (Riccardi and Eichner 1986).

The gene for NF1 has been mapped by two laboratories to the long arm of chromosome 17 (Barker et al. 1987; Seizinger et al. 1987*b*). Linkage analyses in multigenerational families with DNA markers from chromosome 17 have identified DNA fragments that are very tightly linked to the NF1 gene (Diehl et al. 1987; Fain et al. 1987; Pericak-Vance et al. 1987; Seizinger et al. 1987*a;* Stephens et al. 1987; Upadhyaya et al. 1987; vanTuinen et al. 1987; White et al. 1987). In addition, two individuals with NF1 have been identified with chromosomal translocations with breakpoints in the region of the NF1 gene (Schmidt et al. 1987; Ledbetter et al. 1989; Menon et al. 1989). Using DNA from these patients, two laboratories have

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further localized the NF1 gene within a region of approximately 600 kb (Fountain et al. 1989; O'Connell et al. 1989). Since the current work was initiated, the NF1 gene has been isolated (Cawthon et al. 1990; Viskochil et al. 1990; Wallace et al. 1990). Its relatively large size, more than 300 kb, may explain the high rate of sporadic occurrence of this disease (Huson et al. 1989).

We have observed losses of DNA markers from the NF1 region of the long arm of chromosome 17 in DNA from malignant tumors from patients with NF1, compared with DNA from nontumor tissue from the same patients (Skuse et al. 1989). Further, in hereditary cases, we have found that the NF1 allele remaining in the tumor was derived from the affected parent (authors' unpublished observations). These data and those of others (El-Azouzi et al. 1989) suggest that malignant tumors in NF1 probably arise as the result of the homozygous deficiency of a tumor-suppressor gene. This contention is supported by the recent identification of regions of homology between the predicted NF1 amino acid sequence and that of the GTPase-activating protein (Xu et al. 1990) and the yeast inhibitor of *ras* proteins IRA1 and IRA2 (Buchberg et al. 1990; Xu et al. 1990).

The loss of heterozygosity for DNA markers tightly linked to the NF1 locus has been observed in malignant tumors but not in neurofibromas. In order to investigate the possibility that neurofibromas are polyclonal in origin, we have analyzed neurofibromas from female NF1 patients by using an X chromosome–specific probe, from the PGK gene, which detects an RFLP.

The Lyon hypothesis describes the process of X-chromosome inactivation in mammalian females (Lyon 1972). At some early stage of embryogenesis one X chromosome is inactivated in each cell. Whether the chromosome inactivated is maternally derived or paternally derived appears to be random. Subsequently, all progeny of a given cell have an inactivated X chromosome of the same parental origin. Therefore, a female is a mosaic of cells with either the maternally or paternally derived X chromosome inactivated. In view of this, a tumor that has arisen from more than one cell (i.e., one which is polyclonal or multicellular in origin) should be composed of a mixture of cells with maternally or paternally derived X chromosomes inactivated. In contrast, a tumor that has arisen from only one cell (i.e., one which is monoclonal or unicellular in origin) should have inactivated the X chromosome of the same parental origin in every cell. Earlier studies by Fialkow et al. (1971) using glucose 6-phosphate dehydrogenase isozymes as markers provided evidence that neurofibromas were polyclonal in origin.

We have reinvestigated this question by using recently developed DNA-based methods. Using both an X chromosome–specific probe which detects an RFLP and the restriction endonuclease *Hpa*II to differentiate between the maternal and paternal active X chromosomes, we have analyzed neurofibromas from females with NF1. We obtained evidence which suggests that the neurofibroma arises as the result of a mutation in a single cell.

Material and Methods

Neurofibromas

The neurofibromas included in the present study were obtained from patients with authentic von Recklinghausen neurofibromatosis and from either the University of Rochester Medical Center (fresh) or other medical centers (shipped frozen). Most were cutaneous in origin, but one was retroperitoneal. None of these were plexiform neurofibromas. The diagnosis of neurofibroma was made on the basis of typical gross and microscopic features. Skin and other adjacent normal tissues were removed as completely as possible prior to DNA preparation.

DNA Probes

The DNA probes used in the present study were provided by the investigators listed in table 1. The human DNA component of each recombinant plasmid was purified by agarose gel electrophoresis followed by ultrafiltration through Ultrafree MC filters (Millipore) according to the instructions provided by the manufacturer.

Table 1

DNA Probes Used in Present Study

Probe	Contributor	Reference
A10-41	D. Barker	Barker et al. 1987
EW301	D. Barker	Fain et al. 1987
HHH202	R. White	Nakamura et al. 1987
Beta 8-2	D. Barker	Personal communication
VAW215-R3	D. Barker	Personal communication
EW206	D. Barker	Fain et al. 1987
EW207	D. Barker	Fain et al. 1987
PGK 800	B. Vogelstein	Vogelstein et al. 1987

Southern Blot Analyses

Southern blot analyses were carried out as described elsewhere (Skuse et al. 1989) by using the method of Southern (1975), with some modifications. For analysis of loss of heterozygosity, DNA was resolved on agarose gels (1% in Tris-acetate EDTA buffer) and transferred by capillary blotting to Zeta Bind membrane (AMF Cuno) in 10 × SSC. Following transfer, the membranes were soaked in 0.4 N NaOH for 1 min, then were neutralized in 0.2 M Tris (pH 7.5), 2 × SSC, and then air-dried. Analyses with the PGK 800 probe involved resolution on 1.5% agarose gels (in Tris-borate EDTA buffer) and included a preannealing step with sheared salmon sperm DNA (Sealey et al. 1985). Transfer was carried out in 0.4 N NaOH followed by neutralization in 2 × SSC for 30 min with one change of buffer. Transfer membranes were dried at 37°C for 1 h and baked at 80°C for 30 min. Hybridizations were carried out as described elsewhere (Skuse et al. 1989), except that reactions with PGK 800 included BLOTTO (Vogelstein et al. 1985) in the hybridization mixture. DNA probes were labeled with ^{32}P-dCTP by random primer labeling according to the instructions supplied with the labeling kit (Boehringer Mannheim).

Analysis of Loss of Heterozygosity

To investigate loss of heterozygosity, tumor and nontumor DNA from the same patient were compared following digestion with a restriction endonuclease and Southern blotting (Skuse et al. 1989). The blots were hybridized with sequences previously localized to the region of the NF1 locus (listed in table 1).

Analysis of Clonal Origin

For neurofibromas donated by females with NF1, clonality was investigated using the multiple-step method of Vogelstein et al. (1985, 1987). First, those heterozygous for the RFLP detected by the X-linked phosphoglycerokinase gene probe, PGK 800, were identified by RFLP analysis of nontumor tissue. DNA from their neurofibromas was then digested with the restriction endonucleases *Pst*I and *Bst*XI or with *Bgl*I, *Eco*RI, and *Bgl*II. Second, one-half of each reaction mixture was subjected to further digestion with *Hpa*II. The detected alleles carry additional recognition sites for the methylation-sensitive enzyme *Hpa*II, so that the allele derived from the active X chromosome is digested by *Hpa*II while the one from the hypermethylated, inactive X chromosome is resistant to further digestion. Thus, comparison of the DNA samples digested with and without *Hpa*II revealed whether (1) the same X chromosome is inactivated in every cell of the neurofibroma, which would indicate monoclonal origin (evidenced by loss of one or the other allelic fragments), or (2) the neurofibroma is a mixture of (*a*) cells carrying an active maternally derived X chromosome and (*b*) cells carrying an active paternally derived X chromosome (evidenced by comparable loss of both allelic fragments).

Analysis of Tissue Composition

Each neurofibroma was sectioned, stained with hematoxylin-eosin, and examined by a single experienced neuropathologist who estimated the percentage of nontumor tissue present in the sample of tumor from which DNA was purified.

Results

Loss of Heterozygosity

We analyzed 19 sample pairs, each consisting of DNA from a neurofibroma and peripheral blood from a NF1 patient (male or female), by using seven DNA probes localized to chromosome 17. The results are presented in table 2. We did not observe a loss of heterozygosity in any of the sample pairs analyzed with any of the probes.

PGK Heterozygosity

We analyzed nontumor tissue from 30 unrelated female NF1 patients for heterozygosity detected by a probe from the PGK locus. Eight individuals heterozygous for this RFLP were identified. DNA from neurofibromas from these individuals was subjected to further analysis.

Clonal Origin of Tumor Tissue

DNA from neurofibromas from the eight individuals heterozygous for the PGK RFLP was subjected to analysis by *Hpa*II digestion. Analyses of each of the eight patients are shown in figure 1 and summarized in table 3. All of the neurofibromas analyzed demonstrated a monoclonal pattern—namely, a disproportionate reduction of one or the other allele after *Hpa*II digestion; "disproportionate" refers to a greater than 14-fold reduction in density of one band compared with the other.

Table 2

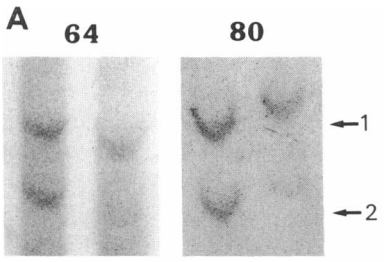
Analysis of Neurofibromas from Female NF1 Patients, by Using Probes for Chromosome 17

Patient Number	Genotype (nontumor tissue:neurofibroma)[a]						
	A10-41	EW301	HHH202	Beta 8-2	VAW215R3	EW206	EW207
73	1,2:1,2	1:1	1:1	1,2:1,2	1,2:1,2	1,2:1,2	1,2:1,2
65	2:2	2:2	1:1	1,2:1,2	1,2:1,2	2:2	1,2:1,2
24	1:1	2:2	1:1	2:2	1:1	1,2:1,2	1,2:1,2
4	1,2:1,2	1,2:1,2	1,2:1,2	1,2:1,2	2:2	1,2:1,2	1,2:1,2
64	1:1	1,2:1,2	1,2:1,2	2:2	1,2:1,2	2:2	1:1
59	1,2:1,2	1:1	1,2:1,2	1,2:1,2	2:2	2:2	1:1
31	1:1	1,2:1,2	1,2:1,2	1,2:1,2	1,2:1,2	2:2	1:1
10	1,2:1,2	1,2:ND	1:1	1:1	1,2:ND	2:2	1:1
21	1,2:1,2	1,2:1,2	1:1	1,2:1,2	1,2:1,2	2:2	1,2:1,2
53	1,2:1,2	1,2:1,2	1:1	1,2:1,2	1,2:1,2	1,2:1,2	2:2
35	1:1	2:2	1,2:1,2	1,2:1,2	1,2:1,2	2:2	1:1
20	2:2	2:2	1,2:1,2	2:2	1,2:1,2	2:2	1:1
90	2:2	1:1	1:1	1:1	1,2:1,2	1,2:1,2	2:2
81	1,2:1,2	1,2:1,2	1,2:1,2	2:2	1,2:1,2	2:2	2:2
77	1,2:1,2	1,2:1,2	1:1	2:2	1,2:1,2	2:2	1,2:1,2
63	1,2:1,2	1,2:1,2	1,2:1,2	1:1	2:2	2:2	1:1
27	ND:1	ND:1	ND:1	1,2:1,2	ND	ND:2	ND:1
80	1,2:1,2	2:2	1,2:1,2	1:1	2:2	1,2:1,2	ND:1
54	1,2:1,2	1,2:1,2	1:1	2:2	1,2:1,2	2:2	1,2:1,2

[a] 1 = presence of larger fragment; 2 = presence of the smaller fragment; ND = not determined.

Clonal Origin of Nontumor Tissue

Nontumor tissue was available from six of the eight patients heterozygous for the PGK RFLP. DNA from these nontumor tissues was subjected to the same analysis as was the DNA from neurofibromas. Five of these six had a proportional reduction of both alleles from *Hpa*II digestion while the sixth had a disproportionate loss of one allele, probably the result of a skewed pattern of X-chromosome inactivation during embryogenesis (Gartler and Linder 1964; Linder and Gartler 1965). The eight neurofibromas included in the present study have been analyzed by our pathology department to determine their tissue composition. Except for patient number 64, who contained approxi-



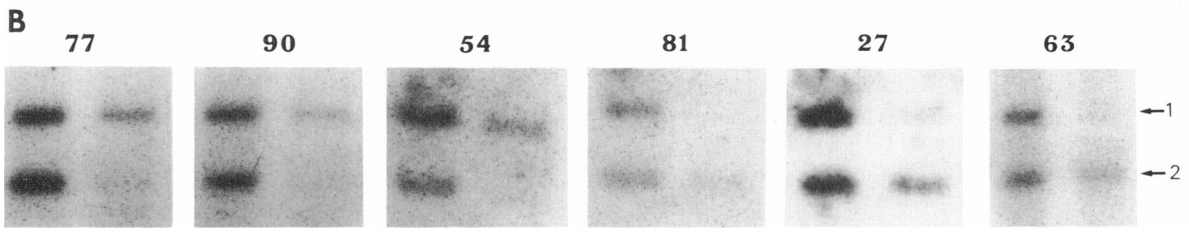


Figure 1 Analysis of clonality of neurofibromas from eight NF1 patients heterozygous for PGK 800. DNA was digested with a mixture of restriction endonucleases, either *Bgl*I, *Eco*RI, and *Bgl*II (*A*) or *Pst*I and *Bst*XI (*B*). Half of each digest was then digested with *Hpa*II (right-hand member of each pair). Above each sample pair is the laboratory patient number. The two expected alleles are indicated by arrows, with "1" indicating the larger allele and "2" indicating the smaller allele.

Table 3

Analysis of Clonal Origin of Neurofibromas

Condition and Patient Number	Fold Reduction by Densitometry[a]	
	Allele 1 (upper band)	Allele 2 (lower band)
Allele 1 chiefly reduced:		
27	15.3	2.1
63	22.2	1.4
81	19.8	2.1
Allele 2 chiefly reduced:		
54	4.5	14.5
64	1.7	>20.0
77	5.6	>20.0
80	1.1	>20.0
90	4.3	38.0

[a] Ratio of band density without *Hpa*II digestion to band density with *Hpa*II digestion.

mately 20% infiltrating normal tissue, all had 0%–10% associated normal tissue.

Analyses of control tissues are shown in figure 2. The first pair (fig. 2*A*) consists of DNA isolated from a normal female's blood, a polyclonal tissue. The alleles present on both X chromosomes are visible and reduced by approximately 50% following digestion with *Hpa*II. The second pair (fig. 2*B*) consists of DNA isolated from a cell line derived from a breast carcinoma, a tumor of monoclonal origin. Both alleles are present before *Hpa*II digestion, but only one, from the hypermethylated inactive X chromosome, remains after *Hpa*II digestion. The third pair (fig. 2*C*) consists of DNA isolated from the peripheral blood of a male. Only one allele is observed from the sole X chromosome and is degraded by digestion with *Hpa*II.

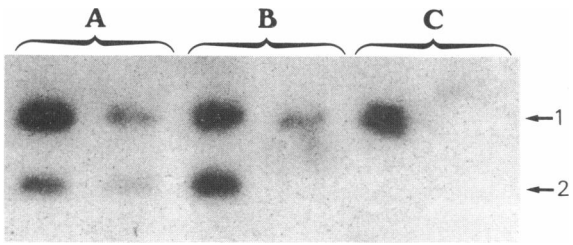


Figure 2 Analysis of clonality of control tissues. A representative autoradiograph is shown for several control samples digested with *Bst*XI and *Pst*I with (right lane of each pair) or without (left lane of each pair) subsequent digestion by *Hpa*II. The first pair (*A*) consists of DNA isolated from a normal female's peripheral blood. The second pair (*B*) consists of DNA isolated from cells derived from a breast carcinoma cell line. The third pair (*C*) consists of DNA isolated from the peripheral blood of a male. The two expected alleles are indicated as in fig. 1.

Discussion

Elsewhere we have reported a loss of heterozygosity for several markers on chromosome 17 in malignant tumors occurring in patients with NF1 (Skuse et al. 1989). Here we report no similar losses detected in benign neurofibromas in a larger series of samples and with additional chromosome 17 probes. This failure to observe losses may have several explanations. The first is that our samples may have been infiltrated with nontumor tissue; normal cells would not be expected to have a loss and might therefore obscure any losses in tumor cells. The eight neurofibromas analyzed in the present study were minimally contaminated with nontumor tissue. The second explanation is that neurofibromas may be polyclonal in origin, as was reported by Fialkow et al. (1971). The analyses reported here used DNA methods that not only are more sensitive but permit study of a larger number of affected females, because of the three available DNA marker systems (Vogelstein et al. 1987). The data reported here demonstrate that the neurofibromas analyzed are monoclonal in origin. We conclude that neurofibromas in NF1 are true neoplasms in the sense that they arise from a single cell.

The finding of a monoclonal DNA pattern in a type of tumor recognized to contain cells of diverse types requires explanation. First, the resolution of any method which identifies clonality is finite; the possible existence of a clone too underrepresented to be detected cannot be excluded. Second, the initial transforming event might have occurred in several cells all bearing the same active X chromosome. Clearly, the smaller the region of cells containing inactivated X

chromosomes of only maternal or only paternal origin, the more likely is such an occurrence (Linder and Gartler 1965; Woodruff 1988). Third, chromosome loss in tumors is common; it is conceivable that X-chromosome loss might be responsible for an apparent monoclonal pattern. However, if the loss is random, it is not likely that most cells would lose the X chromosome of the same parental origin. Fourth, cells with inactivation of a given X chromosome may have a selective growth advantage; thus a tumor of multicentric origin might evolve a monoclonal DNA phenotype (Raskind and Fialkow 1987). Such a selection process appears to occur in blood cells of heterozygotes for Lesch-Nyhan disease, an X-linked condition (Dancis et al. 1968; Nyhan et al. 1970). Fifth, the tissue analyzed in the present study may have consisted of a higher proportion of Schwann cells whereas the tissue analyzed in the previous study of the cellular origin of neurofibromas (Fialkow et al. 1971) may have had a larger proportion of non-Schwann cells. If the non-Schwann cells re not part of the neoplasm, the polyclonality detected by the glucose-6-phosphate dehydrogenase isozyme assay is explained. Sixth, perhaps the most interesting possibility is that the isozyme analyses and the DNA methods provide answers to different questions and that these answers are not discordant. A monoclonal DNA pattern provides strong evidence of a unicellular origin (subject to the provisos discussed above); a polyclonal isozyme phenotype could result from subsequent development of heterogeneity of either DNA sequence or transcriptional activity. Such a conclusion was reached in the case of parathyroid adenomas which were reported to be polyclonal by isozyme analysis (Fialkow et al. 1977) but were reported to be of unicellular origin by DNA analysis (Arnold et al. 1988).

Recognition of a monoclonal pattern by using an X-linked probe in the analyses presented here implies that our failure to find a loss of heterozygosity with chromosome 17 probes is not due to excessive contamination by normal tissue. The finding that neurofibromas do not show this loss of heterozygosity indicates that neurofibromas arise by a different mechanism than do malignant tumors in NF1 — presumably by either a mutation, at the NF1 locus, not detectable as a loss of heterozygosity or a mutation involving a locus other than the NF1 locus. The wide-ranging manifestations of NF1, as well as the observations by others of the involvement of a chromosome 17p locus in astrocytomas with and without NF1 (El-Azouzi et al. 1989; James et al. 1989), suggest that more than one locus may be involved in malignant tumorigenesis. This is particularly plausible in view of recently published observations of the genetic lesions involved in the evolution of breast carcinomas (Devilee et al. 1989), gliomas (James et al. 1988), and colorectal carcinomas (Fearon et al. 1990).

In bilateral acoustic neurofibromatosis, or NF2, a loss of heterozygosity was reported in six neurofibromas when DNA probes from the NF2 region of chromosome 22 were used (Seizinger et al. 1987*c*; Rouleau et al. 1990). These findings suggest that neurofibromas in NF2 are also monoclonal but may arise by a mechanism involving the NF2 gene.

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