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Linkage Studies with Chromosome 17 DNA Markers in 45 Neurofibromatosis 1 Families

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A locus for von Recklinghausen neurofibromatosis (NF1) has recently been mapped near the chromosome 17 centromere. We have extended these linkage studies by genotyping 45 NF1 families with three DNA probes known to be linked to the chromosome 17 centromeric region. Of 34 families informative for NF1 and at least one of the three probes, 28 families show no recombinants with the disease gene. These data provide additional support for genetic homogeneity of NF1 and for a primary NF1 locus linked to the chromosome 17 centromere. Among the informative families were 7 families with apparent new NF1 mutations. Our data suggest that these mutations are probably at the chromosome 17 NF1 locus.

INTRODUCTION

von Recklinghausen neurofibromatosis (NF1) is an autosomal dominant disorder with an incidence of 1 in 3000 individuals and a high mutation rate estimated at 0.0001 mutation per gamete per generation (Crowe *et al.*, 1956). The diagnostic features are multiple superficial neurofibromas, hyperpigmented skin macules, and iris Lisch nodules, although additional clinical manifestations are often observed (for a recent review see Riccardi and Eichner, 1986). Expression of the disease is variable; both the types of lesions and their severity differ widely even among individuals of a single family (Riccardi, 1981). A locus for NF1 has recently been identified by genetic linkage to DNA probes on chromosome 17 in 23 pedigrees (Barker *et al.*, 1987a; Seizinger *et al.*, 1987). These data show that a locus for NF1 is on chromosome 17 in these families; however, linkage analysis on additional families is necessary to determine if there is more than one NF1 locus.

Since the existence of genetic heterogeneity would have important implications for future diagnostic testing with DNA probes, and isolation and characterization of the NF1 gene(s), we have begun genotyping a large collection of NF1 families with chromosome 17 probes. In this paper we report the results of genotyping 45 NF1-affected families with three chromosome 17 DNA markers. Our data provide additional support for the genetic homogeneity of NF1.

MATERIALS AND METHODS

Family Resouces

The 45 NF1-affected families used in this study were contributed by two centers: 40 families from the Baylor Neurofibromatosis Program (designated BAY) were diagnosed and collected by V.M.R., and 5 families from the University of Michigan (designated UMI) were diagnosed and collected by F.S.C. The 5 UMI families were also used in the study by Diehl *et al.* (this volume). These 45 families comprise 282 individuals, with the children representing 165 meioses, and represent a variety of ethnic backgrounds. The criteria for diagnosis of NF1 are outlined in Riccardi and Eichner (1986) and Riccardi and Cary (1987). Blood drawn from affected and at-risk individuals in these two sets of families was sent to Collaborative Research, Inc., where lymphoblastoid cell lines were established by transformation with Epstein–Barr virus.

The genotypes for the three DNA markers on the NF1 families were combined with the genotypes previously compiled (see below) on 21 multigenerational nondisease families provided by the Centre d'Etude du Polymorphisme Humain (CEPH).

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DNA Isolation and RFLP Typing

High-molecular-weight DNA from lymphoblastoid cell lines was isolated manually as previously described (Schumm *et al.*, 1987) or automatically by a DNA extractor (Model 340A, Applied Biosystems, Inc.). The computer program for the DNA extractor was modified slightly for the following conditions. Isolated nuclei from 30–50 million lymphoblastoid cells were injected into each vessel containing 2.9 ml of 1× lysis buffer (Applied Biosystems, Inc.) and 25 units of proteinase K. Samples were digested for 2 h at 55°C with the vessels mixing at a 140° angle. The samples were extracted twice with 4.5 ml of phenol:chloroform, followed by a single extraction with chloroform (2.7 ml). Sodium acetate (3 *M*) was delivered at 0.33 ml per vessel, followed by 3.1 ml of isopropanol. The precipitated DNA was collected on Precipitette filters (Applied Biosystems, Inc.), washed twice with 70% ethanol, and resuspended in 10 m*M* Tris–hydrochloride (pH 7.5) and 1 m*M* EDTA to a concentration of 250 μg/ml. All reagents for the DNA extractor were purchased from Applied Biosystems, Inc.

Methods for restriction endonuclease digestion, electrophoresis, Southern blotting, hybridization with labeled DNA probes, and isolation and characterization of the two RFLP probes, CRI-L581 and CRI-L946, are described by Donis-Keller *et al.* (1987). The nerve growth factor receptor probe, pE51, detects RFLPs with both *Hin*cII and *Xmn*I (Breakefield *et al.*, 1986); however, we have genotyped only the *Hin*cII RFLP on a subset of 40 of the 45 NF1 families.

Linkage Analysis

To perform two-point linkage analysis between the NF1 locus and the linked markers, we derived, by hand, an expression for the log likelihood function (as a function of the sex average recombination fraction) in each NF1 family, and then evaluated this expression (by computer) for each multiple of 0.01 up to 0.5. Complete penetrance at the NF1 locus was assumed.

Multipoint linkage analysis was performed using the program CRI-MAP (Barker *et al.*, 1987b; Donis-Keller *et al.*, 1987). This program allows rapid maximum likelihood estimation of map distances for many loci simultaneously, but requires the families analyzed to be of "CEPH structure": i.e., having two parents, four grandparents (of which some or all may be missing), and one or more children. To use this program on the NF1 families they were split, where necessary, into "CEPH structure" subfamilies; this splitting results in loss of information in some families, but does not bias the recombination fraction estimates. The data set used for multipoint linkage analysis then consisted of the 21 CEPH families on which linkage data for the markers had previously been compiled (Donis-Keller *et al.*, 1987), together with the data on the (split) NF1 families. The multipoint map was constructed by sequential addition of loci, using a procedure previously described (Barker *et al.*, 1987b; Donis-Keller *et al.*, 1987).

RESULTS

DNA Probes

We have two DNA probes, CRI-L581 and CRI-L946, that were previously mapped to human chromosome 17 by two methods (Donis-Keller *et al.*, 1987). First, CRI-L581 was assigned to chromosome 17 by hybridization to panels of somatic cell hybrids. Second, both probes were linked to two previously localized probes: the chromosome 17 alpha-satellite probe, p17H8, which detects the locus D17Z1 (Willard *et al.*, 1986), and the nerve growth factor receptor probe, pE51 (Breakefield *et al.*, 1986). CRI-L581 and CRI-L946 map near the centromere, with 0% recombination between p17H8 and CRI-L581, and a 3 cM sex-averaged distance between the (p17H8–CRI-L581) cluster and CRI-L946 (Donis-Keller *et al.*, 1987). These three probes, whose unique order could not be determined, mapped 17 cM from the nerve growth factor receptor locus at 17q12 → 17q22 (Donis-Keller *et al.*, 1987). The previously reported linkages between D17Z1 and NF1 (Barker *et al.*, 1987a) and between nerve growth factor receptor and NF1 (Seizinger *et al.*, 1987) indicate that CRI-L581

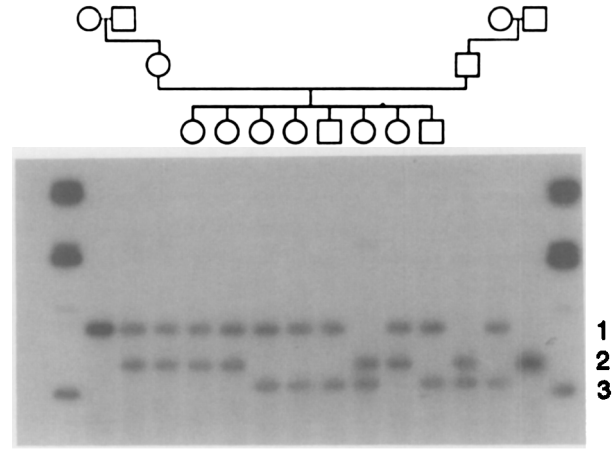

FIG. 1. Inheritance of the RFLP detected by CRI-L581. RFLP probe CRI-L581 displays three alleles on Southern hybridization to DNA digested with *Taq*I from the CEPH reference family 1341. The alleles correspond to fragments of 8.7, 7.5, and 7.0 kb, respectively. The outermost lanes are DNA fragment size markers.

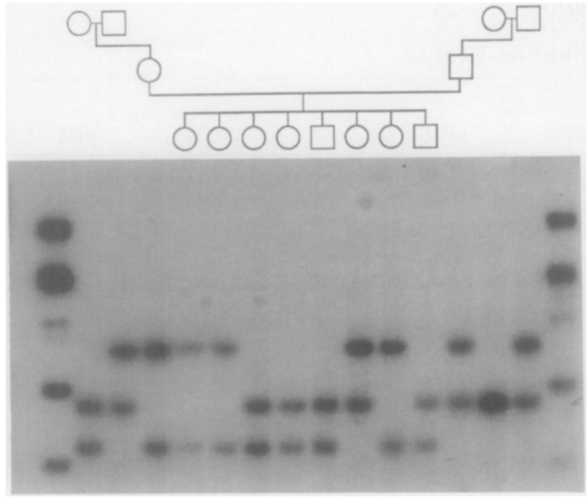

FIG. 2. Inheritance of the RFLP detected by CRI-L946. RFLP probe CRI-L946 displays three alleles on Southern hybridization to DNA digested with *Msp*I from the CEPH reference family 1341. The alleles correspond to fragments of 8.3, 6.3, and 5.2 kb, respectively. The outermost lanes are DNA fragment size markers.

and CRI-L946 should also be linked to the NF1 locus on chromosome 17.

CRI-L581 has a heterozygosity of 0.55 and detects a three-allele restriction fragment length polymorphism (RFLP) with the restriction enzyme *Taq*I as shown in Fig. 1 (Donis-Keller *et al.*, 1987). CRI-L946 has a heterozygosity of 0.43 and detects a three-allele RFLP with the enzyme *Msp*I as shown in Fig. 2 (Donis-Keller *et al.*, 1987).

Linkage Analysis

Genotyping 45 NF1-affected families, comprising 165 meioses, resulted in 34 families informative for at least one of the three probes. Twenty-one families were informative for CRI-L581, 23 families for CRI-L946, and 10 for pE51. Only 3 NF1 families were informative for all three markers. The results of the linkage analysis are presented in Table 1. One obligate crossover in 56 meioses occurred between CRI-L946 and NF1 in family BAY40 (Table 2). Five obligate crossovers in 59 meioses occurred between CRI-L581 and NF1 (Table 2). All individuals with a recombination event between NF1 and either CRI-L581 or CRI-L946 are affected with neurofibromatosis with the exception of the recombinant BAY-43-02 (Table 2).

All families showing recombination events with the disease were examined for possible nonpaternity and/or sample mixup with either one of two probes that detect highly polymorphic DNA sequences: The 3'HVR probe (heterozygosity = 0.93) associated with the HBA1 gene on chromosome 16 (Jarman *et al.*, 1986) or the CRI-L1065 probe (heterozygosity = 0.74) on chromosome 6 (Donis-Keller *et al.*, 1987; Kazazian *et al.*, 1986). The possibility of sample mixup between parents or among siblings was examined by probing the recombinant families with pDP105, a Y-chromosome-specific probe (Disteche *et al.*, 1986). These analyses indicated no sample mixups or nonpaternities had occurred in the six recombinant families. Furthermore, the medical records of all recombinant families were reexamined and in one family, BAY40, the diagnoses of the siblings were verified by repeat physical examination at the Baylor Neurofibromatosis Program Clinic by V.M.R.

Pairwise LOD scores for the three DNA markers were calculated from the genotypic data on NF1 families combined with the previously compiled genotypic data on 21 CEPH families (Donis-Keller *et al.*, 1987). For the markers CRI-L581 and CRI-L946 the maximum LOD score was 32.60 ($\theta = 0.03$), and for markers CRI-L581 and pE51 the maximum LOD score was 10.51 ($\theta = 0.08$). The maximum LOD score of 2.38 ($\theta = 0.18$) was obtained for CRI-L946 and pE51.

We have published a multipoint linkage map of chromosome 17 with genotypic data on 21 CEPH families for the following chromosome 17 probes: myosin (MYH2), the centromeric alpha-satellite probe p17H8, CRI-L581, CRI-L946, and the nerve growth factor receptor probe pE51 (Donis-Keller *et al.*, 1987).

TABLE 1

Pairwise LOD Scores for NF1 and Chromosome 17 Markers

	LOD score at θ of							
Marker	0.0	0.1	0.2	0.3	0.4	$\hat{z}$	$\hat{\theta}$	CI[a]
CRI-L946	$-\infty$	7.14	5.00	2.83	1.01	8.37	0.02	0.0001–0.09
CRI-L581	$-\infty$	5.41	4.42	2.81	1.19	5.43	0.09	0.03–0.20
pE51	4.80	3.62	2.43	1.30	0.41	4.80	0.00	0.0–0.09

Note. $\hat{z}$ = maximum LOD score; $\hat{\theta}$ = recombination fraction at $\hat{z}$.
[a] One LOD unit confidence interval.

TABLE 2

NF1 Families with Recombination Events

Family	Recombinant Individual	NF–L946	NF–L581	NF–pE51	L946–L581	pE51–L946	pE51–L581
BAY21	03 or 04	—	R	—	—	—	I
BAY25	03 or 04	—	R	—	R	—	—
BAY28	03 or 04	—	R	I	—	I	R
BAY40	04	R	—	—	—	—	—
BAY43	02	—	R	—	—	—	—
BAY45	03	—	R	—	—	—	—

Note. R, recombination event; I, informative, no recombination; —, not informative.

When multipoint analysis was performed on the combined data set of CEPH families and NF1 families, the order of the four loci, D17Z1, CRI-L581, CRI-L946, and NF1, could not be uniquely determined. However, the cluster of these four loci mapped with greater than 100:1 odds between myosin (17p13) and nerve growth factor receptor (17q12 → q22).

DISCUSSION

To examine the possibility of genetic heterogeneity in von Recklinghausen neurofibromatosis we have genotyped 45 NF1 families with three DNA probes on chromosome 17: pE51, CRI-L581, and CRI-L946. The results of our linkage analysis show that of 34 families informative for at least one of the probes, 28 families showed no recombinants between the marker and NF1. The maximum LOD scores between NF1 and each of these markers are significant for linkage (Table 1). Although additional NF1 loci cannot be ruled out, these data provide support for the genetic homogeneity of NF1, and for a primary NF1 locus linked to the chromosome 17 centromeric region.

Combining the genotypic data collected on the NF1 families with those previously compiled on the CEPH families (see Materials and Methods) provided a sufficiently large data set for detection of significant differences in female and male recombination frequencies between some pairs of probes. The sex-specific LOD between CRI-L946 and CRI-L581 was 33.90, with θ for females at 0.06 and θ for males at 0.00 ($\chi^2 = 5.98$). The sex-specific LOD between CRI-L581 and pE51 was 13.13, with θ for females at 0.39 compared to $\theta = 0.02$ for males ($\chi^2 = 12.09$). Sex-specific differences in recombination frequencies have previously been reported for the chromosome 17 centromeric region (Barker *et al.*, 1987).

Among the 34 informative families were 7 "sporadic" NF1 families, i.e., families that exhibit a documented absence of NF1 in both parents and all (available) grandparents of an NF1-affected individual. Six of these families yielded positive LOD scores (at $\theta = 0$). Only one recombinant family, BAY25 (Table 2), has a documented sporadic mutation. Although far from conclusive, these data suggest that in sporadic cases of NF1 at least some of the presumably new mutations are probably occurring at the chromosome 17 NF1 locus.

Six NF1 families had one family member with an obligate crossover between NF1 and one of the DNA markers (Table 2). In two of these families there is evidence that a crossover has occurred near the chromosome 17 NF1 locus. In family BAY25 there is a crossover between CRI-L946 and CRI-L581 (Table 2), and a crossover in family BAY28 between pE51 and CRI-L581 (Table 2). These data suggest that families BAY25 and BAY28 are not cases of genetic heterogeneity. For the remaining four recombinant families (Table 2) we have no such evidence; more data are needed to determine whether or not these families are examples of genetic heterogeneity. We conclude, as have others (Barker *et al.*, 1987; Seizinger *et al.*, 1987), that if there is another locus for NF1 besides the one identified on chromosome 17, then it accounts for only a small percentage of cases.

Since very few of the NF1 recombinant individuals are informative for other pairs of loci (Table 2), multipoint linkage analysis cannot as yet determine whether CRI-L946 and CRI-L581 flank the NF1 locus. However, preliminary examination of the two-point data in Table 2 suggests two possible orders: CRI-L946, NF1, CRI-L581 or NF1, CRI-L946, CRI-L581. The order of markers in the NF1 region will become clearer as we isolate and map additional probes on the recombinant families.

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REFERENCES

1. BARKER, D., WRIGHT, E., NGUYEN, K., CANNON, L., FAIN, P., GOLDGAR, D., BISHOP, D. T., CAREY, J., BATY, B., KIVLIN, J., WILLARD, H., WAYE, J. S., GREIG, G., LEINWAND, L., NAKAMURA, Y., O'CONNELL, P., LEPPERT, M., LALOUEL, J.-M., WHITE, R., AND SKOLNICK, M. (1987a). Gene for von Recklinghausen neurofibromatosis is in the pericentric region of chromosome 17. *Science* **236:** 1100–1102.
2. BARKER, D., GREEN, P., KNOWLTON, R., SCHUMM, J., LANDER, E., OLIPHANT, A., WILLARD, H., AKOTS, G., BROWN, V., GRAVIUS, T., HELMS, C., NELSON, C., PARKER, C., REDIKER, K., RISING, M., WATT, D., WEIFFENBACH, B., AND DONIS-KELLER, H. (1987b). A genetic linkage map of 63 DNA markers on human chromosome 7. *Proc. Natl. Acad. Sci. USA* **84:** 8006–8010.
3. BREAKEFIELD, X. O., OZELIUS, L., BOTHWELL, M. A., CHAO, M. V., AXELROD, F., KRAMER, P. L., KIDD, K. K., LANAHAN, A. A., JOHNSON, D. E., ROSS, A. H., AND GUSELLA, J. F. (1986). DNA polymorphism for the nerve growth factor receptor gene excludes its role in familial dysautonomia. *Mol. Biol. Med.* **3:** 483–494.
4. CROWE, F. W., SCHULL, W. J., AND NEEL, J. V. (1956). "A Clinical, Pathological, and Genetic Study of Multiple Neurofibromatosis," pp. 1–181, Thomas, Springfield, IL.
5. DIEHL, S. R., BOEHNKE, M., ERICKSON, R. P., BAXTER, A. B., BRUCE, M. A., LIEBERMAN, J. L., PLATT, D. J., PLOUGHMAN, L. M., SEILER, K. A., SWEET, A. M., AND COLLINS, F. S. (1987). Linkage analysis of von Recklinghausen neurofibromatosis to DNA markers on chromosome 17. *Genomics* **1:** 361–363.
6. DISTECHE, C. M., BROWN, L., SAAL, H., FRIEDMAN, C., THULINE, H. C., HOAR, D. I., PAGON, R. A., AND PAGE, D. C. (1986). Molecular detection of a translocation (Y:15) in a 45,X male. *Hum. Genet.* **74:** 372–377.
7. DONIS-KELLER, H., GREEN, P., HELMS, C., CARTINHOUR, S., WEIFFENBACH, B., STEPHENS, K., KEITH, T. P., BOWDEN, D. W., SMITH, D. R., LANDER, E. S., BOTSTEIN, D., AKOTS, G., REDIKER, K. S., GRAVIUS, T., BROWN, V. A., RISING, M. B., PARKER, C., POWERS, J. A., WATT, D. E., KAUFFMAN, E. R., BRICKER, A., PHIPPS, P., MULLER-KAHLE, H., FULTON, T. R., NG, S., SCHUMM, J. W., BRAMAN, J. C., KNOWLTON, R. G., BARKER, D. F., CROOKS, S. M., LINCOLN, S. E., DALY, M. J., AND ABRAHAMSON, J. (1987). A genetic linkage map of the human genome. *Cell* **51:** 319–337.
8. JARMAN, A. P., NICKOLLS, R. D., WEATHERALL, D. J., CLEGG, J. B., AND HIGGS, D. R. (1986). Molecular characterization of a hypervariable region downstream of the human α-globin gene cluster. *EMBO J.* **5:** 1857–1863.
9. KAZAZIAN, H. H., JR., ORKIN, S. H., BOEHM, C. D., GOFF, S. C., WONG, C., DOWLING, C. E., NEWBURGER, P. E., KNOWLTON, R. G., BROWN, V., AND DONIS-KELLER, H. (1986). Characterization of a spontaneous mutation to a β-thalassemia allele. *Amer. J. Hum. Genet.* **38:** 860–867.
10. RICCARDI, V. M. (1981). von Recklinghausen neurofibromatosis. *N. Engl. J. Med.* **305:** 1617–1627.
11. RICCARDI, V. M., AND CAREY, J. (1987). von Recklinghausen neurofibromatosis and genetic linkage studies: Clinical considerations. *J. Med. Genet.* **24:** 521–522.
12. RICCARDI, V. M., AND EICHNER, J. E. (1986). "Neurofibromatosis: Phenotype, Natural History, and Pathogenesis," pp. 1–36, Johns Hopkins Univ. Press, Baltimore, MD.
13. SCHUMM, J., KNOWLTON, R., BRAMAN, J., BARKER, D., BOTSTEIN, D., AKOTS, G., BROWN, V., GRAVIUS, T., HELMS, C., HSAIO, K., REDIKER, K., THURSTON, J., AND DONIS-KELLER, H. (1988). Identification of more than 500 restriction fragment length polymorphisms by screening random genomic clones. *Amer. J. Hum. Genet.* **42:** 143–159.
14. SEIZINGER, B. R., ROULEAU, G. A., OZELIUS, L. J., LANE, A. H., FARYNIARZ, A. G., CHAO, M. V., HUSON, S., KORF, B. R., PARRY, D. M., PERICAK-VANCE, M. A., COLLINS, F. S., HOBBS, W. J., FALCONE, B. G., IANNAZZI, J. A., ROY, J. C., ST. GEORGE-HYSLOP, P. H., TANZI, R. E., BOTHWELL, M. A., UPADHYAYA, M., HARPER, P., GOLDSTEIN, A. E., HOOVER, D. L., BADER, J. L., SPENCE, M. A., MULVIHILL, J. J., AYLSWORTH, A. S., VANCE, J. M., ROSSENWASSER, G. O. D., GASKELL, P. C., ROSES, A. D., MARTUZA, R. L., BREAKEFIELD, X. O., AND GUSSELLA, J. F., (1987). Genetic linkage of von Recklinghausen neurofibromatosis to the nerve growth factor receptor gene. *Cell* **49:** 589–594.
15. WILLARD, H. F., WAYE, J. S., SKOLNICK, M. H., SCHWARTZ, C. E., POWERS, V. E., AND ENGLAND, S. B. (1986). Detection of restriction fragment length polymorphisms at the centromeres of human chromosomes by using chromosome-specific alpha-satellite DNA probes: Implications for development of centromere-based genetic linkage maps. *Proc. Natl. Acad. Sci. USA* **83:** 5611–5615.