

Molecular analysis of neurofibromatosis type 1 in Turkish families using polymorphic markers

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Neurofibromatosis type 1 (NF1) is a common autosomal dominant disorder characterized by multiple neurofibromas, café-au-lait spots, and Lisch nodules of iris. The NF1 gene is located on chromosome 17q11.2 and encodes an 11-13 kb mRNA containing 60 exons. The NF1 gene product neurofibromin is a large protein of 2818 amino acids which acts as a negative regulator in the ras signal transduction pathway. The disease has a high mutation rate and a wide range of expression. Because of the size and complexity of the gene, the variety of mutations and the need to identify the specific mutation in each family, indirect diagnosis using linked markers has an important part in genetic counseling. We analyzed 10 Turkish families with a total of 28 affected individuals and 34 non-affected relatives using polymorphic sequences, four intragenic and five flanking markers. Intragenic microsatellite markers were highly informative for all families. As a result, prenatal and presymptomatic diagnoses for familial cases are being made available.

Key words: neurofibromatosis type 1, presymptomatic diagnosis, molecular analysis.

Neurofibromatosis type 1 (von Recklinghausen disease) (NF1) is an autosomal dominant disorder affecting 1 in 3,000 to 4,000 newborns. The main clinical features of the disease are the presence of café-au-lait spots (CLS), neurofibromas and Lisch nodules of iris which are present in over 90% of NF1 patients after puberty. Minor features of the disease are macrocephaly and short stature^{1,2}. NF1 is associated with tumors (optic glioma, peripheral nerve malignancy, rhabdomyosarcoma, pheochromocytoma, duodenal carcinoid). Although most features of NF1 do not cause major clinical problems, subcutaneous and plexiform neurofibromas can be disfiguring. In addition, the patients are prone to a number of complications causing significant morbidity and mortality, such as orthopedic (scoliosis, pseudoarthrosis) and cognitive problems³⁻⁵.

Neurofibromatosis type 1 (NF1) gene is located at chromosome 17q11.2. Its conservation through evolution from yeast to mammals supports its biological importance. The protein product of the gene, neurofibromin, belongs to a family of proteins known as GTPase

activating proteins^{6,7}.

The only known function of human neurofibromin is negative regulation of Ras oncogene, suggesting a biologically significant role in intracellular signal transduction⁸. The highest level of neurofibromin expression is in the central nervous system¹.

Neurofibromatosis type 1 (NF1) gene spans 350 kb of genomic DNA, and contains 60 exons. Its mutation rate is higher than observed for most human genes because of its large size, the presence of intragenic repeated sequences, and the probable occurrence of interlocus gene conversion from several NF1-pseudogenes^{1,7}. Almost 50% of NF1 patients are sporadic cases. NNF (National Neurofibromatosis Foundation) International NF1 Mutation Analysis Consortium has recorded 246 NF1 gene mutations, including chromosomal abnormalities, large deletions or insertions, microdeletions or insertions, splicing mutations, and nonsense and missense mutations which do not cluster to any gene region (<http://www.nf.org/nf1/gene/>). Molecular analysis in the NF1 gene should allow

identification of the mutation, if the total gene is analyzed with powerful mutation detection techniques. The mutational spectrum in NF1 gene illustrates the need for multiple complementary techniques by accompanied protein truncation test (PTT) in order to detect the pathogenic lesions^{9,10}. Recently, however, LabCorb has announced that it has discontinued prenatal testing for NF1 by using only PTT, because false-negative results are possible (http://www.nf.org/research_calls/). As a result, the use of traditional approaches for the identification of mutations in NF1 patients is limited. DNA testing for NF1 by linkage analysis may be most useful as an adjunct to clinical diagnosis of NF1 for young children who have not yet developed symptoms and for the fetus. In addition, large deletions in NF1 gene can be obtained with linkage analysis in sporadic or familial cases. Presymptomatic diagnosis is important, because symptoms such as learning disability, bone lesions and tumors can be decreased. Although NF1 patients are diagnosed according to the NIH (National Institute of Health) clinical diagnostic criteria, molecular diagnosis is necessary for prenatal and presymptomatic diagnosis¹¹⁻¹⁴. In our study, four intragenic and five flanking markers were used for linkage analysis (Table I). The use of microsatellites in intron 27 with the highly informative microsatellite of intron 38 covers 50 kb of the gene. In addition, this 50 kb region includes CpG rich exons, which are mutational hot-spots^{15,16}.

The aim of our study was to obtain molecular genetic data for prenatal and presymptomatic

diagnosis in our families with NF1 by using polymorphic markers.

Material and Methods

Ten families with at least two affected members who presented to Hacettepe University Pediatric Clinic, a total of 62 subjects including 28 affected individuals, were studied. All were diagnosed according to the NIH criteria^{11,17}. After informed consent was obtained from all families, DNA was extracted from peripheral blood¹⁸. Segregation analysis was performed using five extragenic restriction fragment length polymorphisms (RFLPs)^{12,19-21} and four intragenic microsatellite markers^{15,22-24} as shown in Table I. Three RFLPs (p11.3C4.2, pEW207, p2F9.8) were excluded in the last five families. All polymorphisms were analyzed by polymerase chain reaction (PCR) based methods. PCR products digested with appropriate restriction enzymes were electrophoresed on 3% agarose gels containing ethidium bromide and were visualized under ultraviolet fluorescence. PCR products of microsatellite markers were electrophoresed on 7% denaturing polyacrylamide gels and DNA bands were detected by silver staining.

Results

We performed segregation analysis in 10 families using polymorphic markers. Recombinations and double recombinations were obtained with three flanking markers (pEW206, pEW207, p2F9.8). The other RFLPs and microsatellite markers were informative for the families. Thus, prenatal diagnosis could

Table I. Restriction Fragment Length Polymorphisms and Microsatellite Markers

Marker Reference	Genetic position and distance from NF1 gene	Fragment size (bp)	Restriction enzyme	
p11.3C4.2	Proximal, <3cM	1080	MspI	16
pHHH202	Proximal, <0.6 cM	306	RsaI	17
Alu (AAAT repeat)	Intragenic, intron 27b	407-395	–	19
IVS27AC28.4 (AC repeat)	Intragenic, intron 27b	219-207	–	20
EVI-20 (CA repeat)	Intragenic, intron 27b	203-191	–	21
IVS38GT53.0 (GT repeat)	Intragenic, intron 38	187-171	–	22
pEW206	Distal, 2.3 cM	349	MspI	18
pEW207	Distal, 7.4 cM	587	Bgl/II	16

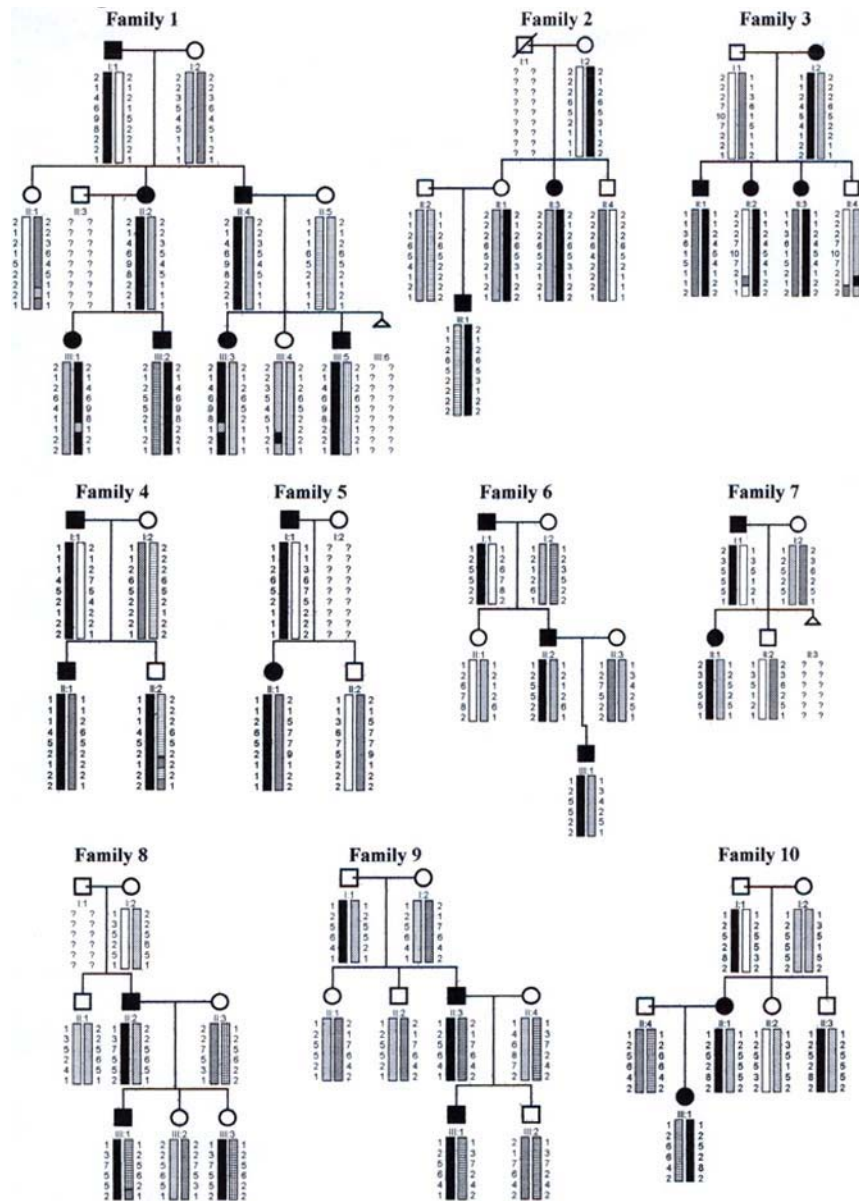


Fig. 1. Pedigrees of families. Marker order (families 1-5): p11.3C4.2, pHHH202, Alu, IVS27AC28.4, EVI-20, IVS38GT53.0, pEW206, p2F9.8, pEW207. Marker order (families 6-10): pHHH202, Alu, IVS27AC28.4, EVI-20, IVS38GT53.0, pEW206.

be offered to all families. Brief clinical and laboratory data of these subjects are listed below and pedigrees are shown in Figure 1.

Family 1

The three-generation family has 11 affected individuals. The proband (III-3), who was nine years old, had optic glioma and plexiform neurofibromas in addition to skin findings and Lisch nodules, and also several affected relatives on her father’s side. Recombinations were

obtained in the family which was informative for the extragenic marker pHHH202 and four intragenic microsatellites, Alu, IVS27AC28.4, EVI-20, IVS38TG53.0.

Family 2

The proband (III-1) who was six years old, had a plexiform neurofibroma, CLS and axillary freckling; his mother had only three CLS smaller than 1.5 cm. The maternal aunt had segmental neurofibroma. The maternal grandmother had

affected chromosome but not NF1 symptoms. The family was informative for two extragenic markers, p2F9.8 and p11.3C4.2, and for four intragenic microsatellites, EVI-20, IVS38GT53.0, Alu, IVS27AC28.4. Segmental and classical NF1 are in the same family. It is a rare situation, and further analysis for this family is underway.

Family 3

The two-generation family had four affected members. Three siblings were affected, and one of them (II-1), who had a cerebellar tumor, was 16 years old. The mother (II-2) had a plexiform neurofibroma. In addition all affected members had skin findings. Recombinations were obtained in the family which was informative for two extragenic markers, p11.3C4.2 and pHHH202, and for four intragenic microsatellites, Alu, EVI-20, IVS27AC28.4, IVS38GT53.0.

Family 4

The two-generation family had two affected members. The proband (II-1), who was nine years old, had CLS and axillary freckling. His father had a plexiform neurofibroma with CLS. His brother, who was five years old, had the affected chromosome, but no NF1 symptoms. Genetic counseling was given concerning periodic controls. Double recombinations were obtained in the family which was informative for two extragenic markers, p11.3C4.2, and pHHH202, and two intragenic microsatellites, IVS38GT53.0 and IVS27AC28.4.

Family 5

The two-generation family had two affected members. The proband (II-1), who had CLS and axillary freckling, was two years old. Her father had cutaneous neurofibromas with skin findings. The family was informative for four extragenic markers, p11.3C4.2, pEW206, pEW207, p2F9.8, and four intragenic microsatellites, EVI-20, Alu, IVS27AC28.4, IVS38GT53.0.

Family 6

Three members in three successive generations had CLS and axillary freckling. The proband (III-1) was six years old. The family was informative for the extragenic marker pEW206 and for four intragenic microsatellites, Alu, IVS27AC28.4, IVS38GT53.0, EVI-20.

Family 7

The two-generation family had two affected members. The proband (II-1) who was six years old, had plexiform neurofibromas, and her father had cutaneous neurofibromas along with CLS and axillary freckling. This family was informative for the extragenic marker pHHH202 and three intragenic microsatellites, IVS38GT53.0, Alu, EVI-20. Prenatal diagnosis could also be offered.

Family 8

The three-generation family had two affected members. The proband (III-1), who was three years old, had a plexiform neurofibroma, CLS and axillary freckling. His father had plexiform and cutaneous neurofibromas. The proband's eight-month-old sister had a chromosome conferring a high risk, but no NF1 symptoms. Genetic counseling was given concerning periodic controls. Recombination was obtained in the family which was informative for extragenic markers pEW206 and pHHH202 and for four intragenic microsatellites, Alu, EVI-20, IVS27AC28.4, IVS38GT53.0.

Family 9

The proband (III-1), who was 11 years old, had multiple CLS. His father had plexiform and cutaneous neurofibromas. This family was informative for two extragenic markers, pHHH202 and pEW206, and intragenic microsatellites, IVS27AC28.4, EVI-20, Alu, IVS38GT53.0. Prenatal diagnosis could be offered.

Family 10

The proband (III-1), who was two years old, had multiple CLS. Her mother had cutaneous and plexiform neurofibromas. The proband's maternal grandfather and uncle had a high-risk haplotype, but no NF1 symptoms. NF1 symptoms were first seen in proband's mother. The family was informative for four intragenic microsatellites, Alu, IVS27AC28.4, EVI-20 and IVS38GT53.0.

In addition, novel alleles were found at intragenic microsatellites Alu and EVI-20 (located in intron 27b): a 392 bp allele of Alu and 215 bp, 213 bp, 209 bp, and 207 bp alleles of EVI-20.

Discussion

We performed segregation analysis in 10 families using five extragenic and four intragenic polymorphic markers. In all the families, the haplotype in linkage with the mutation could be identified with a minimum of three and maximum of five informative markers. Families were more informative for intragenic microsatellites than for extragenic RFLP markers. For instance, the microsatellite marker IVS38GT53.0 was informative in all families. Because of the high recombination possibility, we excluded two of them in the last five families. This analysis uses intragenic microsatellites that cover about 50 kb of the gene, as well as extragenic markers on both sides of the gene to check for recombination events. Moreover, microsatellites are highly polymorphic and are also useful for detecting large deletions in sporadic cases. Deletions in intron 27 were obtained with linkage analysis in two sporadic cases by Natacci et al.²⁵. The demonstration of absence of heterozygosity at multiple polymorphic loci in the NF1 gene could identify individuals who harbor large gene deletion. Likewise, this analysis could prove useful in somatic NF1 mutation analysis for allelic loss (LOH) in tumor tissue^{14,25,26}. The data obtained in this study indicate that allelic segregation analysis with intragenic NF1 polymorphic markers is a valuable tool for genetic analysis. The availability of similar intragenic markers covering the whole gene should be useful in the process of identifying NF1 mutations for familial and sporadic cases.

Novel alleles were found in this study: a 392 bp allele of Alu repeat and 207 bp, 209 bp, 213 bp and 215 bp alleles of EVI-20. The latter has already been reported in the Italian population with a frequency of 0.017 based on 89 Italian unrelated subjects²⁵. As indicated by Natacci et al.²⁵, novel alleles in linkage analysis could be highly informative markers for further molecular diagnosis.

Our results support the value of molecular methods in improving diagnostic accuracy in NF1, as observed in our family 8 where a young member was found to carry the markers linked to a mutant allele although skin finding had not yet, developed. We have already given presymptomatic diagnosis for this family. On

the other hand, ruling out the diagnosis of NF1 is also possible, as in the member of family 2 (II-3) who had three small CLS+.

Neurofibromatosis type I (NF1) is a disorder predisposing to malignancies, and skeletal or vascular abnormalities. The availability of presymptomatic and prenatal diagnosis allows early detection and close follow-up for potential clinical problems. In the present study we optimized the methods for linkage analysis in our familial cases and demonstrated the efficacy of this method in prenatal and preventive approaches to familial NF1. Our next goal is to detect mutations in our sporadic cases.

REFERENCES

1. Shen MH, Harper PS, Upadhyaya M. Molecular genetics of the neurofibromatosis type 1 (NF1). *J Med Genet* 1990; 33: 2-17.
2. North KN. Clinical aspects of neurofibromatosis 1. *Eur J Pediatr Neurol* 1998; 2: 223-231.
3. Rasmussen SA, Friedman JM. NF1 gene and neurofibromatosis 1. *Am J Epidemiol* 2000; 151: 33-40.
4. Ruggieri M, Packer RJ. Why do benign astrocytomas become malignant in NF1? *Neurology* 2001; 56: 827-829.
5. Li J, Perry A, James CD, Gutmann DH. Cancer-related gene expression profiles in NF1-associated pilocytic astrocytomas. *Neurology* 2001; 56: 885-890.
6. Cawthon RM, Veiss, R, Xu, G. A major segment of neurofibromatosis type 1 gene: cDNA sequence, structure and point mutations. *Cell* 1990; 62: 193-201.
7. Li Y, O'Connell P, Breidenbach HH. Genomic organization of neurofibromatosis type 1 gene (NF1). *Genomics* 1995; 25: 9-18.
8. Zwarthoff EC. Neurofibromatosis and associated tumour suppressor genes. *Pathol Res Pract* 1996; 192: 647-657.
9. Messiaen LM, Callens T, Mortier G. Exhaustive mutation analysis of the NF1 gene allows identification of 95% of mutations and reveals a high frequency of unusual splicing defects. *Hum Mutat* 2000; 15: 541-555.
10. Osborn MJ, Upadhyaya M. Evaluation of the protein truncation test and mutation detection in NF1 gene: mutational analysis of 15 known and unknown mutations. *Hum Genet* 1999; 327-332.
11. Gutmann DH, Aylsworth A, Carey JC. The diagnostic evaluation and multidisciplinary management of neurofibromatosis type 1 and neurofibromatosis type 2. *JAMA* 1997; 278: 51-57.
12. Clementi M, Boni S, Mammi I, Favarato M, Tenconi R. Clinical application of genetic polymorphism in neurofibromatosis type 1. *Ann Genet* 1996; 39: 92-

- 96.
13. Gasparani G, Grifa A, Origone P, Coviello D, Antonacci R, Rocchi M. Detection of a neurofibromatosis type 1 (NF1) homologous sequence by PCR: implications for the diagnosis and screening of genetic diseases. *Mol Cell Prob* 1993; 7: 415-418.
 14. Origone P, Bonioli E, Panucci E, Costabel S, Ajmar F, Coviello DA. The Genoa experience of prenatal diagnosis in NF1. *Prenat Diagn* 2000; 9: 719-724.
 15. Lazaro C, Gaona A, Estivill X. Two CA/GT repeat polymorphism in intron 27 of the human neurofibromatosis type 1 gene. *Hum Genet* 1994; 93: 351-352.
 16. Boulandet EG, Pantel J, Cazeneuve C. NF1 gene analysis focused on CpG-rich exons in a cohort of 93 patients with neurofibromatosis type 1. *Hum Mutat* 2000; 16: 274-275.
 17. National Institutes of Health Consensus Development Conference. Neurofibromatosis: Conference Statement. *Arch Neurol* 1988; 45: 575-578.
 18. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nuc Acid Res* 1988; 16: 1215.
 19. Ward K, O'Connell P, Carey JC, et al. Diagnosis of neurofibromatosis type 1 by using tightly linked flanking DNA markers. *Am J Hum Genet* 1990; 46: 943-949.
 20. Ainsworth PJ, Rodenhiser DI. Rapid nonradioactive detection by PCR of pHHH202/RsaI RFLP linked to neurofibromatosis type 1. *Am J Hum Genet* 1991; 49: 1098-1099.
 21. Fain PR, Barker DF, Goldar DE. Genetic analysis of NF1 : identification of close flanking markers on chromosome 17. *Genomics* 1987; 1: 340-345.
 22. Xu G, Nelson L, O'Connell P, White R. An alu polymorphism intragenic to the neurofibromatosis type 1 gene (NF1). *Nuc Acid Res* 1991; 19: 3764.
 23. Charrow J, Listernick R, Ward K. Autosomal dominant multiple café-au-lait spots and neurofibromatosis type 1. evidence of non-linkage. *Am J Med Genet* 1993;