

Genotype Analysis of the *NF1* Gene in the French Canadians From the Québec Population

Lijuan Fang,¹ Nader Chalhoub,¹ Wentican Li,³ Josué Feingold,⁴ June Ortenberg,⁵ Bernard Lemieux,² and Jean-Paul Thirion^{1*}

¹Département de Microbiologie et d'Infectiologie, Faculté de Médecine, Université de Sherbrooke, Sherbrooke, Québec, Canada

²Département de pédiatrie, Faculté de Médecine, Université de Sherbrooke, Sherbrooke, Québec, Canada

³Laboratory of Statistical Genetics, Rockefeller University, New York, New York

⁴INSERM-393-Hôpital Necker, Paris, France

⁵Department of Genetics, The McGill University-Montreal Children's Hospital Research Institute, Montreal, Canada

We genotyped 19 *NF1* families from the French Canadians of the Québec population with six intragenic polymorphic markers including 2 RFLPs (*EcoRI* and *RsaI*) and 4 microsatellites (IVS26-2.3, IVS27AC28.4, IVS27AC33.1, and IVS38GT53.0). Genotype analysis indicated families 7610 and 7473 bear deletions. In Family 7610 the deletion removed the entire *NF1* gene except exons 1 to 4b. The breakpoint of the deletion is located between exons 4a and 4b. The deletion 7473 was derived from the maternal chromosome and exons 1 to 5 were deleted. The breakpoint of the deletion is located between exons 7 and 13. Their phenotypes are reported. The allele frequencies of microsatellites IVS27AC28.4 and IVS38GT 53.0 are compared to previously reported data from Caucasians, including Spanish and Italians. The difference is statistically significant ($P < 0.0036$) for marker IVS27AC 28.4 between the Québec French Canadian and the Italian population. © 2001 Wiley-Liss, Inc.

KEY WORDS: neurofibromatosis type 1; *NF1*; genotype analysis; microdeletion; French Canadians

INTRODUCTION

Neurofibromatosis type 1 (*NF1*) is an autosomal dominant disorder that afflicts about 1 in 3,500 individuals. *NF1* is characterized by café-au-lait spots, Lisch nodules and fibromatous skin tumors. Patients display assorted medical complications including learning disabilities and malignancy. The *NF1* gene spans about 350 kb at 17q11.2 with 60 exons and has a high mutation rate of 1×10^{-4} /gamete/generation leading to approximately 50% sporadic cases.

Patients with submicroscopic deletions are remarkable for their severe phenotypes including facial anomalies, early onset (before age 10) of cutaneous neurofibromata, and variable cognitive deficits ranging from mild learning disabilities to mental retardation. [Kayes et al., 1992, 1994; Wu et al., 1995, 1997a,b; Leppig et al., 1997]. Haploinsufficiency for neurofibromin is the likely molecular basis of *NF1*. Theoretically, early age at onset of neurofibromagenesis could be attributed to: 1) deletion of the *NF1* gene alone; 2) co-deletion of *NF1* and one of the three genes (*EV12A*, *EV12B* and *OMG*) of unknown function that are embedded in intron 27b; 3) co-deletion of *NF1* and contiguous gene(s); or 4) dysregulation of a gene at the deletion breakpoint. It is unlikely, however, that neurofibromin haploinsufficiency alone could account for early onset of tumorigenesis [Dorschner et al., 2000].

We genotyped 19 *NF1* families from the Québec population and investigated deletion mutations, allele frequency distribution, linkage, and linkage disequilibrium (LD) by using 2 RFLPs (*EcoRI* at intron 1, and *RsaI* at exon 5) and four microsatellites (IVS26-2.3, IVS27AC28.4, IVS27AC33.1, and IVS38GT53.0) (refer to Fig. 5). These four microsatellites are distributed along an approximately 65 kb of the gene [Li et al., 1995]. Linkage disequilibrium among polymorphic marks in the *NF1* region has been previously reported [Jorde et al., 1993; Messiaen et al., 1993; Purandare et al., 1996; Valero et al., 1996]. We report on two

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*Correspondence to: Dr. Jean-Paul Thirion, Département de Microbiologie et d'Infectiologie, Faculté de Médecine, Université de Sherbrooke, Sherbrooke, QC J1H 5N4, Canada.
E-mail: jpthir@courrier.usherb.ca

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families of Quebec French Canadian origin bearing deletions that are not classic microdeletions as described previously.

MATERIALS AND METHODS

Families and Patients

Patients met National Institute of Health NF1 diagnostic criteria [Stumpf et al., 1988]. A total of 15 familial cases and four sporadic cases (45 NF1 patients and 40 normal individuals) from Québec were investigated. Informed consent and genomic DNA [Sambrook et al., 1989] were obtained from all individuals. Sixteen families were two-generation and three were three-generation. Out of the 19 families, two families (7610 and 7473) were partially deleted for the *NF1* gene. Their phenotypes were described as follows.

With Family 7610 (Fig. 1), the mother (I-1) and son (II-1) were diagnosed with NF1. When examined at age 36, the mother (I-1) had slight hypertension. She had numerous café-au-lait spots larger than 15 mm and bilateral axillary freckles. Many café-au-lait spots around the neck were excised for cosmetic reasons. Multiple subcutaneous nodules and neurofibromas, but no plexiform neurofibromas were observed. Bilateral axillary freckles, Lisch nodules and a mild scoliosis of the dorsal spine were also present. She developed arterial hypertension at 33 years of age. Detachment of the interior part of the retina was observed surgically. The rest of the examination was normal.

The son (II-1) is the product of the only pregnancy of this woman (I-1). The pregnancy and the labor were both normal, but a single convulsion occurred on the boy's second day of life. One week after birth, he was noted to have a few café-au-lait spots. When examined at 5 years, he (II-1) had normal growth and development. Physical findings included: head circumference of 56 cm (90% for age 5), more than six café-au-lait spots with sizes larger than 5 mm, bilateral axillary and inguinal freckling, and three subcutaneous neurofibromas, but no Lisch nodules. Although school performance was reported normal he manifested some pronunciation difficulties. The rest of the examination was normal. The boy's 62-year-old grandfather (not included in the genotype analysis) suffered from mild hypertension and prostate dysfunction. On physical examination he had multiple café-au-lait spots, iris Lisch nodules, axillary and inguinal freckling and numerous subcutaneous neurofibromas. The complete pedigree shows 11 members had NF1 and four have passed away. The clinical examinations of the other members were not available for the present study.

In Family 7473 (Fig. 3), the woman (II-1) and her two daughters (III-1 and III-2) were diagnosed with NF1. The woman (II-1) had multiple congenital café-au-lait spots. Her first neurofibroma appeared during puberty. A few isolated neurofibromas appeared on the neck after the birth of her second child. When examined at age 36, she had multiple Lisch nodules, but no other physical or intellectual complications. Her first daughter (III-1) was born with multiple café-au-lait spots and left leg bowing. At age 4 she developed axillary freckling and Lisch nodules, but no neurofibromas. When examined at age 9 the leg bowing had evolved into a tibial pseudoarthrosis. Her younger daughter (III-2) had multiple congenital café-au-lait spots, and an extensive glioma of the optic nerves, chiasm and optic tracts first documented on MRI at age 18 months. Subsequently, she developed bilateral axillary and inguinal freckling and Lisch nodules, but no neurofibromas. She required chemotherapy at age 4 for aggressive growth of the glioma with resultant precocious puberty and visual decline. At age 7, she had no indication of learning disability.

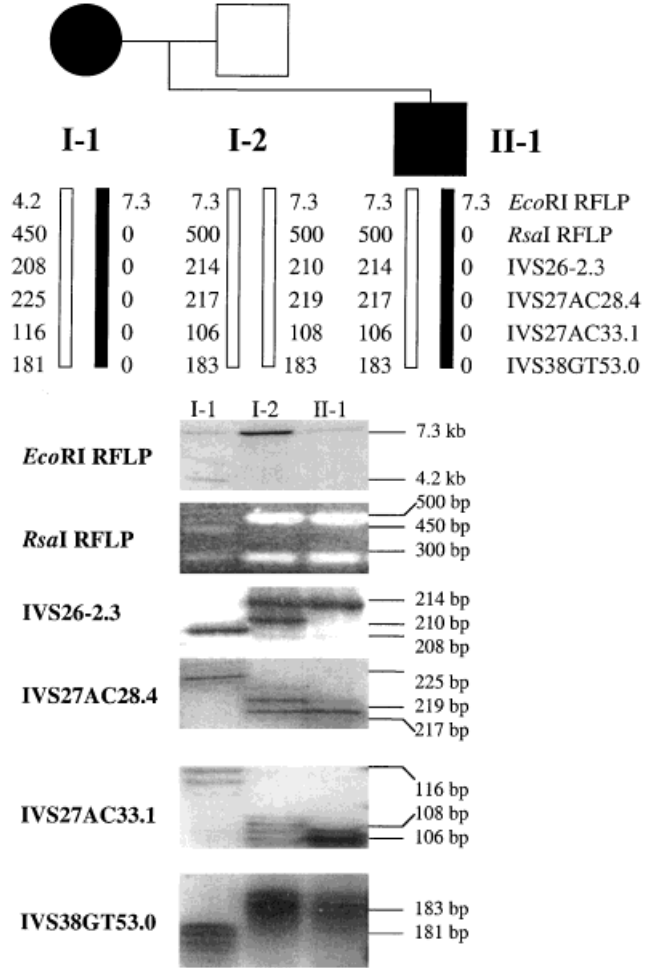


Fig. 1. Pedigree and haplotype of families 7610. Haplotypes were determined by analysis of six polymorphic loci. Microsatellite fragments and *RsaI* polymorphism were indicated as bp at loci along each chromosome. *EcoRI* polymorphism was indicated as fragments in kb. The deleted loci were indicated as 0. The NF1 patients and the mutation-carrying chromosomes are in black.

Southern Blotting and Detection of *EcoRI* Polymorphism

Southern blotting was carried out with 7 cDNA probes, GE2, FF13, FF1, FB5D, P5, and B3A [Sambrook et al., 1989; Wallace et al., 1990; Fang et al., 1999]. Digestion of FF13-876 bp (nt 535-1411,

GenBank accession number M82814) with *HinfI* gave the FF13-625 bp probe (nt 786–1411, GenBank accession number M82814) that was used to fine localization the deletion 7610 breakpoint. The FF13-625 bp fragment covers 12 bp of exon 4b and exons 4c to 9 (nt 798–1411, GenBank accession number M82814) (Fig. 2B).

Detection of Polymorphisms

The *EcoRI* polymorphism is at intron 1 (position 123768, GenBank accession number AC027793). It was determined by Southern blotting [Reyniers et al., 1993]

with probe GE2 (nt 215–616, GenBank accession number M82814). The *RsaI* polymorphism at exon 5 was determined as described [Hoffmeyer and Assum, 1994]. The 4 microsatellites loci are at intron 26 [IVS26-2.3, Bahuau et al., 1996], intron 27b [IVS27AC28.4, Lázaro et al., 1994; IVS27AC33.1, Bahuau et al., 1996] and intron 38 [IVS38GT53.0, Lázaro et al., 1993]. The size of each fragment was determined by a ladder constructed with a pUC18 sequence. At marker IVS27 AC28.4, because we used different primers (Table I) from previous reports [Lázaro et al., 1994], to compare our alleles with those from previous reports, we subtracted 10 nucleotides from each allele. For example,

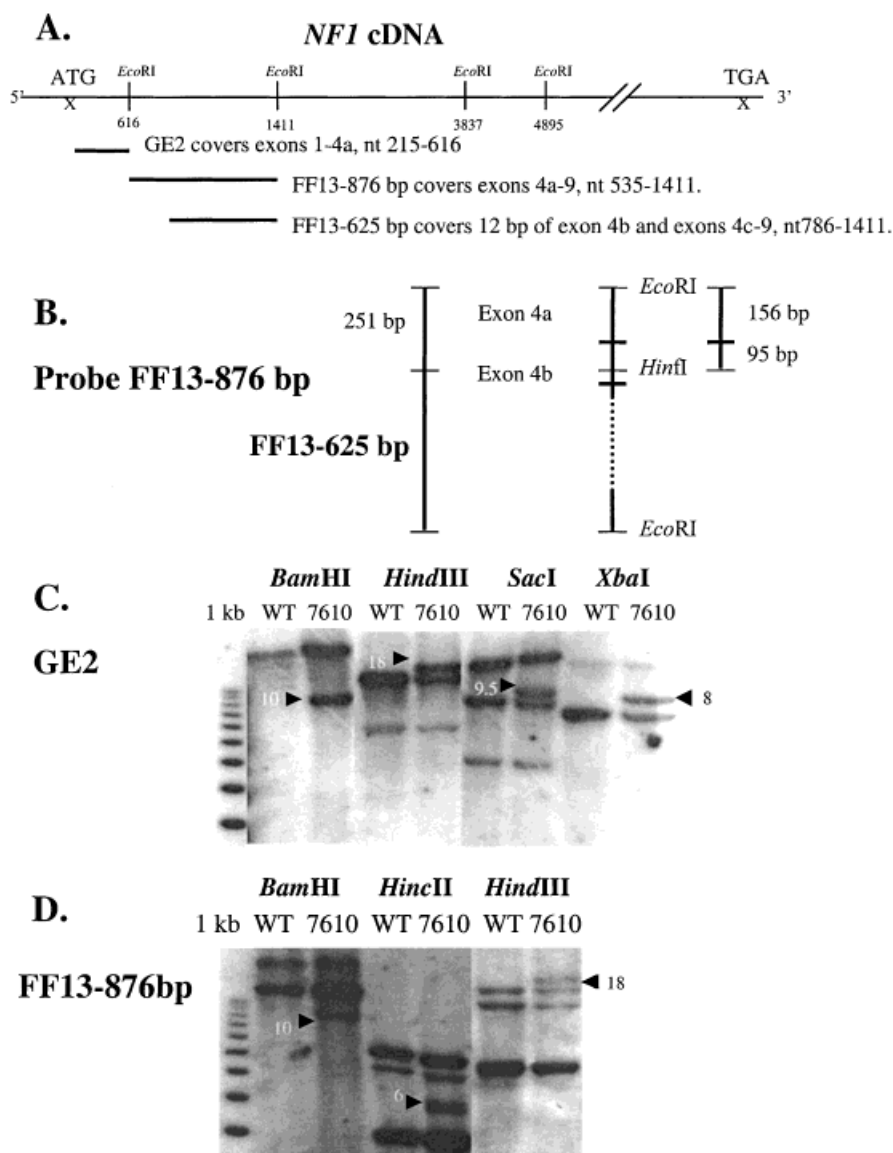


Fig. 2. Southern blot analysis of deletions 7610. Genomic DNA of patient II-1 from Family 7610 and wild-type (WT) was digested with restriction enzymes as indicated and hybridized with probes GE2 (C) or FF13-876 bp (D). The approximate positions of probes GE2, FF13-876 bp, and FF13-625 bp related to the *NF1* cDNA are as depicted (A). FF13-876 bp covers exons 4a to exon 9 (B). FF13-625 bp was made from FF13-876 bp by

digestion with *HinfI* that removes 156 bp of exon 4a and 95 bp of exons 4b from FF13-876 bp. FF13-625 bp covers 12 bp of exon 4b and exons 4c to 9 (B). Vertical lines represent *EcoRI* sites (A) whose nucleotide positions are indicated at the bottom according to GenBank accession number M82814. Extra bands are indicated with arrowheads and the band sizes are indicated as kb.

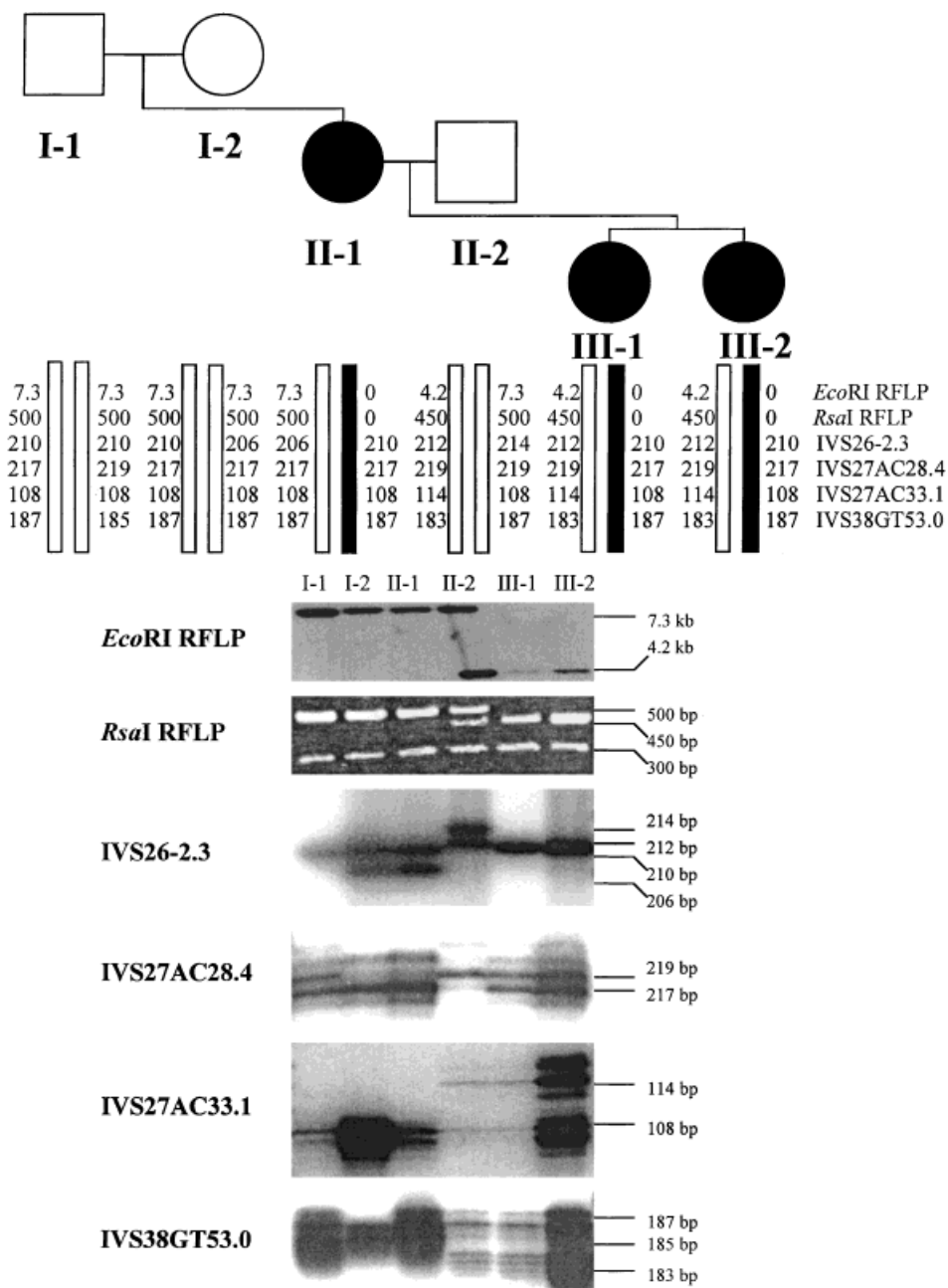


Fig. 3. Pedigree and haplotype of families 7473. For other explanations see Figure 1.

the allele 1 with size of 219 bp for IVS27AC28.4 (Tables II, III) from the Italian population is correspondent to the allele 1 with size of 229 bp in our original data. Tables II and III show the sizes of each allele after subtraction of 10 bp at marker IVA27AC28.4. The PCR was performed in 20 µl total volume with 10 pmol of each primer (Table I), standard PCR buffer, 100 ng genomic DNA and 0.5–1 U of Taq polymerase, and labeled with [α -³²P]dCTP. The reaction was performed for 30 cycles with denaturation at 94°C for 30 sec, extension at 72°C for 30 sec. The annealing temperatures were as described in Table I.

The products were subject to electrophoresis on 8% acrylamide-urea denaturing gels.

Statistical Analysis

Haplotypes were constructed with the GENEHUNTER program [Kruglyak et al., 1996] assuming a minimum number of recombinations. All families were informative at the marker loci, and for each marker the disease-bearing chromosome could be identified.

The allele frequency was calculated with alleles only from parents and married-ins. Married-ins are French

TABLE I. Conditions and Sequences of the Microsatellite Primers

Locus	Allele size (bp)	Tandem repeat	Primers	Annealing temperature (°C)
IVS26-2.3	204–226	(TAGA)(TAGG) (TAAGA)	Forward 5'-AGGCCAGGAGTTCAAGACCA-3' Reverse 5'-ATGAGCCACTGTGCCCAATC-3'	58
IVS27AC28.4	215–231	(CA)	Forward 5'-TGAAGTATGCAGTTTCCAG-3' Reverse 5'-GGCTAAGTGTAACGCAAAG-3'	55
IVS27AC33.1	104–116	(CA)	Forward 5'-TAGATTATATGGGACAGAAAATG-3' Reverse 5'-CTTGAGGTGATGACAGGATG-3'	55
IVS38GT53.0	171–189	(CA)/(GT)	Forward 5'-CAGAGCAAGACCCTGTCT-3' Reverse 5'-CTCCTAACATTTATTAACCTTA-3'	52

Canadians. Children do not provide independent samples, because they derive their alleles from their parents. In total, 79 parents and married-ins were used in the analysis. The frequency distribution of alleles on disease-bearing and non-disease-bearing chromosomes was analyzed across all 19 pedigrees. Affected individuals bearing the disease haplotype from 19 families were selected for analysis. This gave rise to a total of 27 affected haplotypes. The 52 unaffected

haplotypes were taken as controls for the normal allele frequency (104 chromosomes).

Linkage analysis between the disease and markers was carried out using the programs LINKAGE [Terwilliger and Ott, 1994] and GENEHUNTER [Kruglyak et al., 1996] to ensure that the regions being focused on were indeed related to the disease. LINKAGE program was used for two-point analysis and GENEHUNTER for multi-point analysis. Linkage disequilibrium

TABLE II. Alleles Sizes, Frequency Distribution (Overall, Normal, and NF1), and Observed Heterozygosity for Each Microsatellite

Microsatellite	Alleles	Size (bp)	Allele number	Overall	Normal (total = 52)	NF1 (total = 27)	Observed heterozygosity
IVS26-2.3	1	226	8	0.1013	0.0962	0.1111	0.86
	2	222	11	0.1392	0.0962	0.2222	
	3	218	4	0.0506	0.0385	0.0741	
	4	216	4	0.0506	0.0769	0.0000	
	5	214	5	0.0633	0.0577	0.0741	
	6	212	10	0.1266	0.0962	0.1852	
	7	210	20	0.2532	0.2885	0.1852	
	8	208	7	0.0886	0.0962	0.0741	
	9	206	9	0.1139	0.1346	0.0741	
	10	204	1	0.127	0.0192	0.0000	
IVS27AC28.4	1	219	2	0.0253	0.0192	0.0370	0.81
	2	217	12	0.1519	0.1346	0.1852	
	3	215	12	0.1519	0.1346	0.1852	
	4	213	1	0.0127	0.0192	0.0000	
	5	211	7	0.0886	0.0577	0.1481	
	6	209	24	0.3038	0.3077	0.2963	
	7	207	18	0.2278	0.2692	0.1481	
	8	205	3	0.0380	0.0577	0.0000	
IVS27AC33.1	1	116	18	0.2278	0.2115	0.2593	0.75
	2	114	8	0.1013	0.1346	0.0370	
	3	112	1	0.0127	0.0192	0.0000	
	4	110	5	0.0633	0.0385	0.1111	
	5	108	30	0.3797	0.3846	0.3704	
	6	106	16	0.2025	0.1923	0.2222	
	7	104	1	0.0127	0.0192	0.0000	
IVS38GT53.0	1	189	5	0.0633	0.0577	0.0741	0.82
	2	187	17	0.2125	0.2308	0.1852	
	3	185	24	0.3038	0.2500	0.4074	
	4	183	7	0.0886	0.1154	0.0370	
	5	181	10	0.1266	0.1154	0.1481	
	6	177	3	0.0380	0.0385	0.0370	
	7	175	3	0.0380	0.0385	0.0370	
	8	173	7	0.0886	0.0962	0.0741	
	9	171	3	0.0380	0.0577	0.0000	

TABLE III. Comparison Between Previously Reported Allele Frequencies With Those of the Present Study

Microsatellites	Alleles	Size (bp)	Frequency Québec ^a	Frequency 1 ^{b,e}	Frequency 2 ^c	Frequency 3 ^d
IVS27AC28.4	1	219	0.0192	0.012	0.0333	0
	2	217	0.1346	0.045	0.0458	0.057
	3	215	0.1346	0.012	0.0500	0
	4	213	0.0192	0.081	0.1042	0.023
	5	211	0.0577	0.525	0.5792	0.204
	6	209	0.3077	0.210	0.1208	0.534
	7	207	0.2692	0.105	0.0667	0.125
	8	205	0.0577	0	0	0.057
IVS38GT53.0	1	189	0.0577	0	0	0.082
	2	187	0.2308	0.013	0.0420	0.225
	3	185	0.2500	0.230	0.2773	0.041
	4	183	0.1154	0.115	0.210	0.236
	5	181	0.1154	0.180	0.2353	0.214
	6	179	0	0.115	0.1681	0.020
	7	177	0.0385	0.089	0.0840	0.020
	8	175	0.0385	0.038	0.0252	0.041
	9	173	0.0962	0	0.0168	0
	10	171	0.0577	0.220	0.1303	0.091

^abased on 52 Québec French Canadian, present study.

^bbased on 43 Caucasians for IVS27AC28.4 [Lázaro et al., 1994].

^cbased on 125 Spanish [Valero et al., 1996].

^dbased on 44 Italians [Natacci et al., 1999].

^ebased on 39 Caucasians for IVS38GT53.0 [Lázaro et al., 1993].

between markers was determined by a Monte Carlo version of the Fisher's exact test by constructing the joint count table of alleles at two markers from the haplotype data. All other possible joint count tables with the same column and row margins can be in principle enumerated. From the distribution of these tables, *P*-value of any test statistic can be calculated. We randomly sampled 100,000 tables to determine an approximate *P*-value.

RESULTS

Identification and Characterization of Two Deletions

We genotyped 19 NF1 French Canadian families from the Québec population including 85 individuals (45 affected and 40 unaffected) and 170 chromosomes (data not shown). Two deletions were detected by genotype analysis.

Haplotype and segregation analysis of Family 7610 (Fig. 1) reveal that both the mother (I-1) and the son (II-1) are hemizygous for markers at exon 5 (*RsaI* RFLP), introns 26, 27b, and 38, and heterozygous at the *EcoRI* RFLP locus at intron 1. When the genomic DNA of patient II-1 was digested with *Bam*HI, *Hind*III, *Sac*I or *Xba*I, extra bands of about 10, 18, 9.5, 8 kb were detected respectively with the GE2 probe in the mutants (Fig. 2C). When compared with the wild-type, the same extra bands of 10 kb with *Bam*HI and 18 kb with *Hind*III were also detected with FF13-876 bp probes (Fig. 2D). Probe GE2 covers exons 1 to 4a and overlaps with probe FF13-876 bp by 82 bp in exon 4a. Probe FF13-876 bp covers exons 4a to 9. To fine localize

the breakpoint, we made the FF13-625 bp probe by digestion of FF13-876 bp with *Hinf*I to remove 156 bp of exon 4a and 95 bp of exon 4b from FF13-876 bp. The short probe FF13-625 bp covers 12 bp of exon 4b and exons 4c to 9 (Fig. 2B). No abnormal band (blots not shown) was detected with probe FF13-625 bp. This indicates that the breakpoint is between exons 4a and 4b (nt 535–786, GenBank accession number M82814). In this family, the entire *NF1* gene except exons 1 to 4a was deleted (Fig. 5).

With Family 7473 (Fig. 3), markers at introns 26, 27 and 38 of the mother (II-1) and the two daughters (III-1 and III-2) are heterozygous. Examination of the segregation of the *RsaI* RFLP locus (exon 5) indicates that the mother (II-1) is either homozygous or hemizygous because of the presence of a 500 bp and the absence of a 450 bp fragment. Examination of the same *RsaI* locus of the two daughters (III-1 and III-2) indicates that the mother is hemizygous, therefore suggesting a deletion at this locus. The deletion was confirmed by the analysis of *EcoRI* RFLP. The mother (II-1) and the two daughters (III-1 and III-2) were again apparently homozygous at the *EcoRI* loci, with the mother having a 7.3 kb and the two daughters 4.2 kb fragments. Segregation analysis indicated that the 4.2 kb fragments of the daughters came from their father (II-2). This suggests that the mother and the two daughters are hemizygous. This indicates that the deletion covers at least exons 1 to 5 and ends before exon 26. Then, genomic DNA from one member (II-1) of the Family 7473 was analyzed by Southern blotting. Extra bands were detected in the mutant with probe FF1 (Fig. 4) with *Bgl*I and *Bgl*II. We know that probe FF1 covers exons 7 to 13. Thus, the deletion of Family

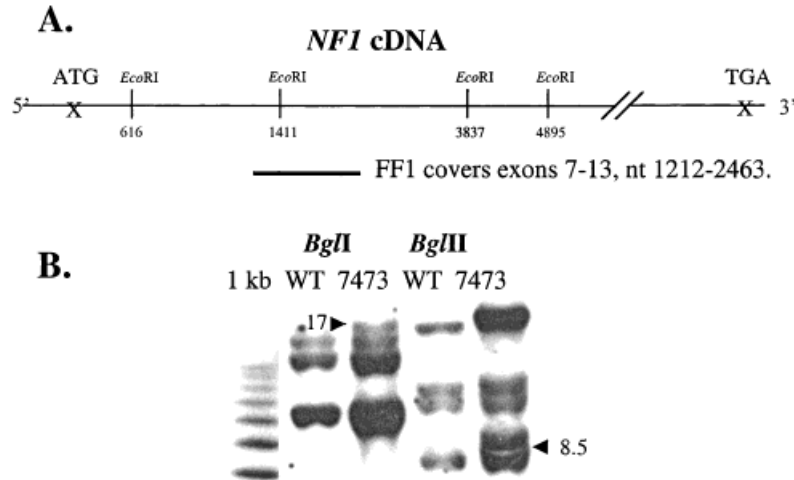


Fig. 4. Southern blot analysis of deletions 7473. Genomic DNA of patient II-1 from Family 7473 and wild-type (WT) was digested with restriction enzymes as indicated and hybridized with probe FF1 (B). The approximate positions of probe FF1 related to the *NF1* cDNA are as depicted (A). Vertical lines represent *EcoRI* sites whose nucleotide positions are indicated at the bottom according to GenBank accession number M82814. Extra bands are indicated with arrowheads and the band sizes are indicated as kb.

7473 removes exons 1 to 7. The breakpoint of the deletion is located between exons 7 and 13 (nt 1212–2463, GenBank accession number M82814) (Fig. 5).

Allele Distribution, Linkage and Linkage Disequilibrium Analysis

Because chromosomes of the mother (I-1) and the son (II-1) of Family 7610 (Fig. 1) were deleted for this part of *NF1* gene covering the four microsatellites, only 166 chromosomes are informative for the four microsatellites. The heterozygosity values of the microsatellites were ranging from 0.75–0.86 (Table II). No significant difference is observed among frequencies between *NF1*-bearing chromosomes and normal chromosomes.

The primers (Table I) used in our study to amplify the microsatellite markers are as described in Methods and Materials. Because for marker IVS27AC28.4 our primers are different from the primers used by other groups, we adjusted the fragment length of each allele of IVS27AC28.4 by subtraction of 10 bp for comparison. At microsatellite IVS27AC28.4, the allele distribution is significantly different from previous reports (Table III) with $P < 0.0036$ between the Québec population and the Italian population [for the Italian population refer to Natacci et al., 1999]. We also detected the allele 8 with size of 205 bp that was reported in the Italian population. Notice that this allele had the same frequency in the Québec French Canadian and the Italian populations. At microsatellite IVS38GT53.0, a

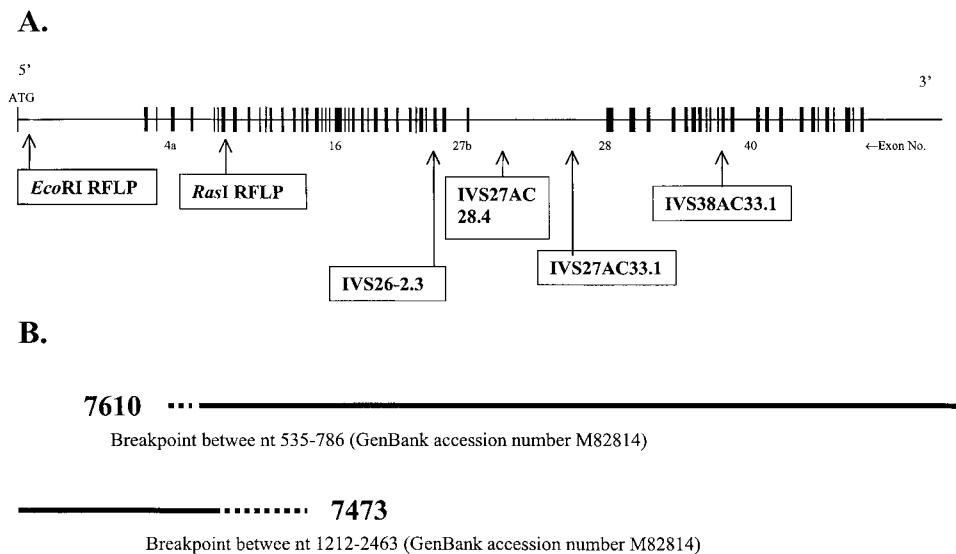


Fig. 5. Approximate extension and distribution of the *NF1* deletions. The *NF1* gene is as depicted (A). The exons are indicated as boxes on the thin horizontal line (representing introns). Genomic locations of polymorphic markers used in haplotyping are as indicated. In the two deletions (B), dot lines indicate the breakpoint and black lines indicate the deleted regions. The diagram is not quite to scale.

significant difference was also observed between our results and those previously reported [Lázaro et al., 1993; Valero et al., 1996; Natacci et al., 1999]. We detected the allele 1 (size 189 bp) reported in the Italian population and the allele 9 (size 173 bp) reported in the Spanish population. Both alleles (allele 1 and allele 10) had significantly different frequencies from the Québec population. Because this is the first report on allele frequencies at microsatellites IVS26-2.3 and IVS27 AC33.1 we could not compare them with data from others.

Except for the marker pair IVS26-2.3 and IVS27 AC33.1, all other marker pairs show significant linkage disequilibrium. A linkage analysis using the program LINKAGE under a dominant model (penetrance values are $P(DD) = P(D+) = 0.9$, $P(+++) = 0.003$) indicates a strong linkage for all four markers with a LOD score of 3.29, 2.97, 3.64, and 2.76 respectively at recombination fraction $\theta = 0.01$. Multipoint linkage analysis using GENEHUNTER program gives a LOD score of 4.35. A three-generation pedigree with 10 members contributes 32% of the LOD score; a two-generation pedigree with six members contributes 20% of the LOD score. Five pedigrees out of 18 contribute 84% of the LOD score, whereas eight other pedigrees each contributes less than 2% of the LOD score.

DISCUSSION

Approximately 70% *NF1* mutations are either frameshift or nonsense mutations. They can be detected with the protein truncation test or other PCR based methods, but large deletions cannot. Large deletions that represent up to 5% of the mutations are best identified by genotype analysis that can possibly detect deletions of even a single marker [Rasmussen et al., 1998]. Two deletions from 19 Québec families were thus detected. One deleted the entire *NF1* gene except exons 1 to 4b and the other deleted exons 1 to 7 (Fig. 5). These two deletions are not classic *NF1* microdeletions as reported previously.

In our study, deletion 7473 is derived from the maternal chromosome. This agrees with previous reports [Lázaro et al., 1996; Upadhyaya et al., 1996, 1998; Ainsworth et al., 1997; Rasmussen et al., 1998; Correa et al., 1999] in that gross deletions are predominantly of maternal origin and result from an unequal crossover in maternal meiosis I. It has been postulated that the mutations of maternal origin are generated by intrachromosomal rearrangements, whereas the paternal mutations originate by interchromosomal rearrangements [Correa et al., 1999, 2000].

For *NF1*, a genotype-phenotype correlation has not been identified except for the deletions of the entire gene that are commonly associated with severe phenotypes such as learning disability/mental retardation, dysmorphic features, multiple neurofibromas, and developmental delay. The presence of a deletion cannot, however, be predicted solely on the basis of the clinical phenotype [Kayes et al., 1992, 1994; Riva et al., 1996, 2000; Upadhyaya et al., 1996, 1998; Cnossen et al., 1997; Leppig et al., 1997; Tonsgard et al., 1997; Valero

et al., 1997; Wu et al., 1997b]. Evidence indicates, however, that large *NF1* deletions might not always be associated with unusual clinical features [Rasmussen et al., 1998].

In our study, the clinical features of these two deletions did not show any distinct manifestations. Although neurofibromas were found, no learning disability/mental retardation, dimorphic features, or developmental delay was diagnosed.

These deletions in the two families are considerably interesting. Unlike most of the other deletions so far described, these do not encompass the entire *NF1* gene and flanking sequences on both sides. The lack of a severe and complex *NF1* phenotype is of particular interest. It provides support for the hypothesis that this severe phenotype results only from a deletion of contiguous gene(s) along with *NF1*. It suggests that the phenotypic effect of deletion is different when the entire gene and flanking sequences are deleted from when a breakpoint occurs within the gene.

Notice that for deletion 7610 (Fig. 5) the proximal breakpoint is within the *NF1* gene, whereas the distal breakpoint may be downstream in a contiguous gene(s). For deletion 7473, the distal breakpoint may be upstream in the *NF1* promoter or a contiguous gene or genes. Phenotypic differences were not obvious between these two deletions. In both cases clinical manifestations were mild. It suggests that deleting either upstream or downstream contiguous sequence may not lead to a severe phenotype. This is in agreement with a previous report on a patient with deletion in the 5'-region of the gene in whom mild clinical manifestations were diagnosed [Hoffmeyer et al., 1994]. Thus, deleting both upstream and downstream sequences on both sides of the *NF1* gene may be needed to cause severe clinical features.

The allele frequencies of microsatellites IVS27 AC28.4 and IVS38GT53.0 are compared to previously reported data (Table III). The difference was statistically significant ($P < 0.0036$) for the marker IVS27 AC28.4 between the Québec population and the Italian population. Microsatellite instability arises from DNA polymerase slippage in cells deficient in DNA mismatch repair. Consequently those cells are at high risk for cancer [Frayling, 1999; Bennett, 2000; Maehara et al., 2001]. Microsatellite instability was observed in neurofibromas [Ottini et al., 1995]. It remains to be answered if the marker instability is due to the high mutation rate of the *NF1* gene or if the marker instability contributes to the high mutation rate.

We observed a strong association between the four microsatellite markers and linkage between the four microsatellites with the disease. A strong LD was observed between five intragenic RFLPs that were revealed by AE25 (covering exons 9–38) with *Bgl*II, *Pvu*II and *Taq*I, Evi2A with *Bam*HI (at intron 27b), and Evi2B with *Eco*RI (at intron 27b) [Messiaen et al., 1993]. LD between the AAAT *Alu* repeats and the microsatellite markers AC127.2 and IVS38GT53.0 were reported [Valero et al., 1996]. A LD with 6 polymorphic markers was also observed [Purandare et al., 1996]. Six internal polymorphisms are all in extremely tight LD

with one another. In fact, five of the six markers are in complete LD, and a high degree of LD was maintained among the seven intragenic markers [Jorde et al., 1993]. In this study, we confirmed the LD at the *NF1* locus with two new microsatellites, IVS26-2.3 and IVS27AC33.1.

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