A genetic study of neurofibromatosis type 1 (NF1) in south-western Ontario. II A PCR based approach to molecular and prenatal diagnosis using linkage

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Abstract

Neurofibromatosis type 1 (NF1) is a common, autosomal dominant genetic disorder with a variety of highly variable symptoms including cutaneous manifestations (such as café au lait spots), Lisch nodules, plexiform neurofibromas, skeletal abnormalities, an increased risk for malignancy, and the development of learning disabilities. The wide clinical variability of expression of the disease phenotype and high (spontaneous) mutation rate of the NF1 gene indicate that careful clinical examination of patients and family members is necessary to provide an accurate diagnosis of the disease. Since very few NF1 mutations have been identified, and with the apparent lack of a predominant mutation in this large, highly mutable gene, molecular diagnosis of NF1 will continue to be based on haplotypes using linkage analysis. Here we report our experiences while providing a molecular diagnostic service for NF1 in the ethnically diverse region of south-western Ontario. Molecular diagnoses with at least one informative probe/enzyme combination are reported for 19 families including two families requesting prenatal diagnosis for NF1. We have augmented the classical Southern based approach to linkage analysis with the use of PCR based assays for molecular linkage. Furthermore, criteria have been established in our laboratory for executing molecular linkage based on heterozygosity values, recombination fractions, and the use of intragenic probes/markers.

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Neurofibromatosis type 1 (NF1) is a common, autosomal dominant genetic disorder with highly variable symptoms which include a variety of cutaneous manifestations (café au lait spots, axillary freckling and dermal neurofibromas), Lisch nodules, plexiform neurofibromas, and skeletal abnormalities.¹ There is an increased risk for malignancy in the form of neurofibrosarcoma, optic glioma, and certain paediatric leukaemias,² and a proportion of affected subjects manifest learning disabilities. Since up to one half of all NF1 cases result from new mutations³ careful clinical examinations are necessary, especially with respect to the parents of affected offspring, so that the

origin of the NF1 mutation (that is, familial inheritance or new mutation) can be established.

The localisation of the NF1 gene to 17q11.2 and the cloning and characterisation of the NF1 gene itself now permit molecular diagnosis of familial NF1.⁴⁻⁶ However, very few mutations have been identified in patients and there is an apparent lack of a predominant mutation in this large, highly mutable gene. This means that molecular diagnosis of NF1 will continue to be based on haplotypes using linkage analysis. We have previously described the population genetics, familial segregation, and molecular linkage of NF1 in families from the ethnically diverse region of south-western Ontario, Canada.⁷ In the present paper we report our experiences in providing a molecular diagnostic service for NF1 in this region of Ontario. This paper also describes our approach to augment classical Southern blot based linkage analysis with PCR based assays which are particularly suitable in prenatal diagnosis.

Materials and methods

SAMPLE ACQUISITION

Families evaluated in this study were referred to our laboratory from the Regional Medical Genetics Centre at the Children's Hospital of Western Ontario and the five other clinical genetics centres throughout Ontario servicing a total population of nine million. Patients were diagnosed by clinical geneticists using the criteria of the NIH Clinical Consensus Symposium.⁸ Criteria for blood collection and shipping instructions for samples were established with all centres before receipt of any samples. Whole venous blood was collected in EDTA coated tubes. Blood was maintained at room temperature and shipped to the laboratory by courier; amniocytes and chorionic villus samples were shipped on ice. All samples were accompanied by accurate family pedigrees. Human genomic DNA was prepared using the modified protocols of Jeanpierre⁹ and Miller *et al.*¹⁰ In all cases, 1 to 2 ml aliquots of whole blood and aliquots of precipitated DNA (under 70% ethanol) were also banked in the laboratory for future use.

MOLECULAR LINKAGE

Molecular linkage analysis was performed either by Southern analysis or PCR based

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assays using the NF1 linked markers listed in table 1. For Southern analyses DNA was digested with the appropriate restriction enzymes (table 1), blotted to nylon membranes, and hybridised with radiolabelled probes as previously described.⁷

For some probes we converted molecular linkage to a PCR based assay, as previously described for the NF1 linked marker pHHH202/*RsaI*.¹¹ PCR primer pairs were generated which flank the polymorphic sites associated with five additional NF1 linked probe/enzyme combinations: pEW206/*MspI*, pF9.8/*RsaI*, p11.3C4.2/*MspI*, pEW207/*BglII*, and pEW207/*HindIII*. For these PCR based analyses reaction parameters recommended by the supplier of *Taq* polymerase (Perkin-Elmer Cetus) were modified so that each amplification reaction (25 µl) included 2.5 µl of 10 × PCR buffer II (Perkin-Elmer Cetus), 10% glycerol (optional for pF9.8), 25 pmol of each primer, 0.625 units of *Taq* polymerase, and 0.5 µg genomic DNA. PCR primer sequences are shown in table 2. Magnesium chloride concentrations optimised for each primer pair include: 1.5 mmol/l $MgCl_2$ (pEW206), 2.0 mmol/l $MgCl_2$ (p11.3C4.2), and 2.5 mmol/l $MgCl_2$ (pEW207 and pF9.8). Samples were overlaid with mineral oil before amplification.

All reaction mixtures were denatured for four minutes at 94°C and then subjected to 30 cycles of PCR. Amplification conditions for p11.3C4.2, pEW206, pF9.8, and pEW207

consist of: primer annealing at 65°C for two minutes, elongation at 72°C for two minutes, and denaturation at 94°C for one minute 10 seconds. In all cases the final amplification cycle was followed by an eight minute extension at 72°C. PCR primers and amplification conditions for analysis of the polyallelic *Alu* repeat marker followed the published protocols^{12,13} (manuscript in preparation).

After amplification RFLPs were detected by digesting 10 µl PCR product in a 15 µl reaction mixture containing 5 to 10 units of the appropriate enzyme (Boehringer Mannheim Canada), 1.5 µl of the appropriate 10 × buffer, and 3.0 µl of water (*MspI* and *RsaI* digests were performed in PCR buffer alone). This mixture was incubated for 90 minutes at 37°C (65°C in the case of *TaqI*). Digestion products were mixed with loading buffer containing marker dye (20% Ficoll 400, 0.1 mol/l EDTA (pH 8), 1% SDS, 0.25% bromophenol blue, and 0.25% xylene cyanol), electrophoresed on a non-denaturing 6% polyacrylamide gel, and then visualised with ethidium bromide. The sizes of PCR products and DNA fragments generated by restriction digestion are given in table 3. Analysis of linkage data generated by Southern and PCR based assays for estimation of genetic risk used the program MLINK with published recombination frequencies for NF1 markers.^{7,14}

Results

FAMILIES

A total of 60 families was referred to the laboratory from the six Regional Medical Genetics Centres of the province of Ontario, 34 of which represent familial cases of NF1. Molecular diagnosis with at least one informative probe/enzyme combination (with a certainty of at least 95%) has been completed for 19 families. Analyses for four families are still in progress since DNA has yet to be received from family members critical for establishing linkage. Eleven files which have been designated inactive represent families who did not wish molecular diagnosis for NF1 or where contact with the families has been lost.

Twenty-four subjects represent apparent sporadic cases of NF1. The higher than expected ratio of familial to sporadic cases of NF1 evaluated by our laboratory (34:24) reflects a selection bias earlier in our research programme when familial cases (with at least two affected subjects) were actively sought to establish linkage relationships between the DNA markers and NF1. New sporadic cases

Table 1 Chromosome 17 DNA markers used for molecular prediction of NF1. Rare allele frequencies are based on founder members of each family. Heterozygosity is calculated based on equilibrium (2pq).

Probe	Enzyme	Relative location	Rare allele frequency	H (observed)	Inf (%)
pHHH202	<i>RsaI</i> *	Proximal	0.36	0.46	11/19 (58)
p11.3C4.2	<i>MspI</i> *	Proximal	0.19	0.31	3/13 (23)
pTH1719	<i>BglII</i>	Proximal	0.40	0.48	3/4 (75)
pEvi2BB	<i>EcoRI</i>	Intragenic	0.37	0.47	3/6 (50)
Alu repeat	—	Intragenic	ND	ND	8/17 (47)
Exon 4	<i>TaqI</i>	Intragenic	ND	ND	1/3 (33)
p11.2C11.7	<i>BamHI</i>	Distal	0.47	0.49	5/7 (71)
pEW206	<i>MspI</i> *	Distal	0.28	0.40	6/18 (33)
p2.F9.8	<i>BglII</i>	Distal	0.33	0.44	4/5 (80)
	<i>TaqI</i>	Distal	0.25	0.38	1/3 (33)
	<i>RsaI</i> *	Distal	0.26	0.38	7/15 (47)
pEW207	<i>BglII</i> *	Distal	0.22	0.34	7/17 (41)
	<i>HindIII</i> *	Distal	0.19	0.31	5/17 (29)

*Probe/enzyme combinations for which a PCR based assay has been developed.
Inf = ratio of families in which probe was informative compared to total families tested.
ND = not determined.

Table 2 Sequence of PCR primers used in linkage analysis of NF1.

Probe (enzyme)	Primer sequence
pHHH202 (<i>RsaI</i>) ¹¹	Forward primer 5'-ATGAACAAGTCAAGGGACAGGCTT-3' Reverse primer 5'-ACTTGCCAAAGGTTACAGGGCTAC-3'
p11.3C4.2 (<i>MspI</i>)	Forward primer 5'-CACAGCTTAGTTTTCAGTGACTCC-3' Reverse primer is 5'-CCTGGTCTACATTTTCAACCTACT-3'
pEW206 (<i>MspI</i>)	Forward primer 5'-TGCAGTGTGGTGCATCATTCACTG-3' Reverse primer 5'-GACAGGGCCAGCCATATTCCTGAT-3'
p2.F9.8 (<i>RsaI</i>)	Forward primer 5'-GAAGTATGAGGTTCTAAGGGAGTA-3' Reverse primer 5'-TCGCCAACAGCTAACATTACGCAG-3'
pEW207 (<i>BglII</i> , <i>HindIII</i>)	Forward primer 5'-AGGTATCAGTCAGGACCTCTTAG-3' Reverse primer 5'-CTGACACTCTGGTTCTGTAATGTG-3'

Table 3 Size of PCR product and fragments detected by polymorphic restriction enzymes.

Probe	Enzyme	PCR product size (bp)	Fragment sizes (bp)
pHHH202	<i>RsaI</i>	306	188/108
p11.3C4.2	<i>MspI</i>	1080	920/160
pEW206	<i>MspI</i>	349	180/169
p2.F9.8*	<i>RsaI</i>	~650	460/400, 60
pEW207	<i>BglII</i>	587	368/219
	<i>HindIII</i>	587	390/197

*Two constant bands are also detected (120, 80 bp).

are now counselled and asked to participate in the continuing research which seeks to identify patient specific NF1 mutations and correlate these mutations with clinical features of NF1. To date, two such patient specific NF1 mutations have been identified by our group in a survey of 50 patients.¹⁵ For two other families referred to the laboratory, the origin of the mutation has yet to be determined owing to incomplete clinical data on particular family members.

MOLECULAR LINKAGE

Molecular linkage analysis of NF1 for 25 families involved the use of a battery of NF1 linked DNA markers flanking the NF1 locus as well as the intragenic marker pEvi2BB/*EcoRI*^{16,16a} (table 1). Observed heterozygosity and allele frequency data for many of the markers are not significantly different from published data.⁷ No recombinants were observed involving NF1 and any markers except F9.8/*RsaI*, where three recombinants were found in 15 meioses. For some analyses we also used two other intragenic markers, either the *TaqI* RFLP detected with a cDNA corresponding to NF1 exon 4^{4,17} or the polyallelic tandem repeat associated with an intragenic NF1 *Alu* sequence^{12,13} (unpublished results). At least one probe/enzyme combination was found to be informative for 19 families; six families require further DNA samples to establish linkage. Fig 1A presents a pedigree and haplotype for one of our NF1 families. Several markers were informative which confirmed the clinical diagnosis of NF1 within this family. Also, a double crossover distal to the NF1 locus is carried by one affected family member. Repeat testing by Southern blotting and PCR based assays for several of the markers confirmed this recombination event. Such double crossover events are expected to be rare in the population (less than 0.5%).

PCR BASED ASSAYS

PCR primers were developed which flank the polymorphic restriction sites detected by the following NF1 linked DNA probes: pHHH202/*RsaI*¹¹, pEW206/*MspI*, pF9.8/*RsaI*, p11.3C4.2/*MspI*, pEW207/*BglII*, and pEW207/*HindIII*.¹⁴ Table 3 lists the size of PCR products and the DNA fragment sizes following digestion with the restriction enzyme which shows the polymorphism. For example, fig 2 shows the results generated by amplification with pEW207 primers and the *HindIII* and *BglII* site genotypes resulting from restriction digestion with each of these enzymes. Such PCR results confirmed the molecular status of all subjects previously established by Southern blot analysis (data not shown). Table 3 lists the six probe/enzyme combinations converted to PCR, the sizes of PCR product, and the DNA fragment sizes following digestion with the appropriate restriction enzyme. In all cases the results of all initial PCR based assays on a panel of control subjects were confirmed by Southern blot analysis.

CRITERIA FOR USING MARKERS FOR LINKAGE ANALYSIS

Using the genetic values for the NF1 linked DNA markers presented in table 1 and our experience in offering molecular diagnosis, a set of criteria has been established which prioritises the DNA markers used for linkage analysis in our laboratory. These criteria attempt to balance the necessity for rapid results (which may be required in the case of prenatal diagnosis), heterozygosity of a marker, and the recombination frequency between a particular marker and NF1. Our prioritised list includes (1) the use of PCR based NF1 linked markers (particularly pHHH202), (2) the use of intragenic polyallelic tandem repeat associated with an intragenic NF1 *Alu* sequence,^{12,13} (3) the use of Southern based intragenic markers pEvi2BB^{16,16a} and *TaqI*/exon 4,¹⁷ and (4) the use of Southern blot analyses with flanking markers (particularly pTH1719 and p11.2C11.7¹⁴).

PRENATAL DIAGNOSIS

Two families requested and received prenatal diagnosis for NF1. In one family the mildly affected mother (presenting with multiple café au lait spots and dermal neurofibromas) had previously given birth to a child with severe NF1 symptoms early in infancy. Prenatal diagnosis using CVS in the subsequent pregnancy indicated a low risk for a fetus carrying the NF1 mutation ($p=0.997$). The parents elected to continue the pregnancy and an apparently unaffected child was born. The second request for prenatal diagnosis came from a couple in which the affected parent had fathered an affected child in a previous marriage (see pedigree and haplotype analysis, fig 1B). Prenatal diagnosis using CVS has been performed in this centre on two subsequent pregnancies resulting from the second marriage. The first prenatal diagnosis indicated a low risk for a fetus carrying the NF1 mutation of paternal origin ($p=0.999$). An apparently unaffected male child was born. Prenatal diagnosis for the second pregnancy indicated a high risk for a fetus carrying the NF1 mutation ($p=0.997$).

Discussion

In this paper we report our experiences while providing a molecular diagnostic service for NF1 in this region of Ontario. Of particular interest, we have augmented the classical Southern blot based linkage analysis with PCR based assays which are particularly suitable in prenatal diagnosis. In conjunction with our PCR based assay for pHHH202,¹¹ six additional NF1 linked probe/enzyme combinations have been converted to PCR. PCR based methods are advantageous in that they can replace or augment the time consuming and more cumbersome Southern analysis with a sensitive, rapid, and non-radioactive approach to linkage analysis. These methods are especially desirable in prenatal diagnosis where gestational age of the fetus is an important

Figure 1 Application of haplotype analysis of NF1 markers in two families: (A) molecular diagnosis and (B) prenatal diagnosis. Sequence of the probe/enzyme combinations used follow table 1. Data which are unavailable are designated -. Localisation and segregation of the NF1 locus is designated where NF=mutation and +=normal. The chromosome carrying the affected haplotype in each pedigree is coloured black.

consideration. Furthermore, this procedure can be adapted to silver stain protocols using the Phastgel system (Pharmacia) thus eliminating the need to handle potentially toxic acrylamide monomer and ethidium bromide.

Our criteria for use of available markers for linkage analysis attempt to balance the need for rapid results and the expense of the test (with respect to labour and resources) with the effectiveness of the particular markers being used (that is, heterozygosity and recombination fre-

quency). As a result, we routinely use PCR based assays in the first stage of our analyses, then use the polyallelic *Alu* repeat assay, followed by Southern based analyses using intra-genic and linked markers (if appropriate). While PCR based assays provide a sensitive and rapid approach to linkage analysis, great care must be taken to ensure accurate results. In order to accomplish this, we assign all workers separate PCR kits with aliquoted reagents and PCR reaction mixtures are pre-

Figure 2 PCR amplification of genomic DNA to reveal the *HindIII* and *BglII* RFLP sites detectable by pEW207. The 587 bp PCR product from genomic DNA of three subjects (lanes 1, 2, 3) was digested with *HindIII* (lanes 4, 5, 6) or *BglII* (lanes 8, 9, 10). *HindIII* digests show the H1 allele lacking the polymorphic *HindIII* restriction site and the H2 allele containing the polymorphic *HindIII* restriction site yielding 197 and 390 bp *HindIII* fragments (lane 5: H2H1 heterozygote; lane 6: H2 homozygote). *BglII* digests show the B1 allele lacking the polymorphic *BglII* restriction site and the B2 allele containing the polymorphic *BglII* restriction site yielding 219 and 368 bp *BglII* fragments (lane 9: B1B2 heterozygote; lane 10: B2 homozygote). Lane 7 shows the pBR322/*HinfI* molecular markers.

pared in a 'clean' room with dedicated micro-pipettors and equipment. No PCR amplified samples are permitted in this work area, and all possible steps are taken in order to eliminate the potential for cross contamination of PCR reaction mixtures. All amplification runs include negative controls (lacking DNA template) and positive controls (containing DNA template from a known heterozygote for a particular probe/enzyme combination). All amplifications and restriction digests are performed in duplicate and in separate runs. Furthermore, to rule out incomplete digestion of PCR product by restriction enzymes, another PCR product (possessing the appropriate restriction site) may be added to the reaction so that complete digestion can be monitored by cleavage of the internal control.

Prenatal diagnosis using CVS tissue requires special consideration in the light of possible maternal contamination. In conjunction with the precautions listed above, we routinely use PCR primers complementary to sequences flanking VNTR loci¹⁸ to rule out maternal contamination of CVS samples. In addition fetal sex is determined using the amelogenin locus,¹⁹ although this information is not routinely included in the clinical report.

Several groups have reported their experiences with presymptomatic detection and prenatal testing of NF1.^{14,20} DNA testing for NF1 by linkage analysis may be most useful as an adjunct to the clinical diagnosis of familial NF1 in young children, for prenatal testing, and when resources available for a complete clinical examination are limited.²¹ In our clinical setting, we have neither formally documented the reasons patients have requested molecular diagnosis nor assessed parental attitudes in our population toward molecular diagnosis and prenatal testing for NF1. However, molecular testing has been used to confirm clinical diagnoses and to ascertain molecular status to allow future reproductive choice.

Two families have requested and received prenatal testing for NF1 and in several cases molecular diagnosis has been requested by families with young children who have yet to exhibit any manifestations of the disease. Formal evaluation of the parental perceptions of molecular testing would be valuable in assessing the impact of molecular diagnosis of genetic diseases such as NF1 on the effective and efficient delivery of limited health care resources.

Attempts by a number of laboratories to characterise family specific mutations within the NF1 gene or to identify common mutations have met with only limited success.^{4,15} Therefore, it is apparent that for the immediate future molecular diagnosis of NF1 will continue to depend on the results of linkage analysis, as more NF1 families request information concerning their genetic status. The augmentation of classical linkage analyses with PCR based assays has, in our experience, facilitated molecular diagnosis of this most common autosomal dominant disease characterised by extensive clinical heterogeneity.

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