A genetic study of neurofibromatosis 1 in south-western Ontario. I Population, familial segregation of phenotype, and molecular linkage

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Abstract
This report is concerned with neurofibromatosis type 1 (NF-1, 17q11.2) in south-western Ontario, an ethnically diverse population derived from multiple immigrations. The population incidence, prevalence, and mutation rates for this disease are similar in most racial groups of this population and are also comparable to earlier reports. NF-1 is one of the most common single gene disorders in this population. The occurrence of the disease is not affected by the birth order or sex of the transmitting parent. The severe manifestation of this disease is statistically related to paternal transmission. Five polymorphic DNA probes (pA1041, pHHH202, pTH1719, NF1, pEW206, pEW207) were evaluated in relation to segregation of NF-1 using appropriate restriction enzymes. The observed heterozygosity was found to be relatively high, ranging from 25% to 55% for all the probes on 17q and flanking the NF-1 gene. We recommend the use of pHHH202/pTH1719 and pEW206 in any linkage analysis for detection of the presence of the NF-1 mutation. For informative families the degree of certainty is as high as 99.5%. Some future modifications may include the use of NF-1 exon specific probes and primers that remain to be evaluated for heterogeneity and heterozygosity among populations.

Neurofibromatosis type 1 (NF-1, von Recklinghausen disease) is one of the most common autosomal dominant disorders with a frequency of approximately 1/3000. Although NF-1 was first noted by Virchow in 1847 and described by von Recklinghausen in 1882, the clinical criteria for its diagnosis were only agreed by an NIH Consensus Panel in 1987. Today the diagnosis of the disease is based on two or more of the following features: six or more café au lait spots; two or more neurofibromas or one plexiform neurofibroma; freckling in the axillary or inguinal regions; optic glioma; two or more Lisch nodules; a distinct osseous lesion such as sphenoid dysplasia or thinning of the long bone cortex, with or without pseudoarthrosis; a first degree relative who meets the above criteria for NF-1. The onset of the expression of NF-1 is age dependent and diagnosis is not always reliable in paediatric cases. Even in adults with familial NF-1 the expression of the disease is highly variable; not all affected subjects show all symptoms and when a given symptom is present in a number of subjects, the severity of the symptom varies greatly. The biological basis for this well recognised phenotypic variability in NF-1 is not known. A number of reports have documented the mode of inheritance, and provided an overall estimate of incidence, prevalence, and mutation rate. Recently Huson et al. reported a detailed genetic analysis of a population in south-east Wales with some interesting features, which emphasises the importance of population studies for this apparently genetically heterogeneous disease.

The genetic mutation(s) responsible for NF-1 appear to involve a single gene locus that maps to chromosome 17q11.2. Many family pedigrees have been analysed with respect to a number of DNA markers that are known to cosegregate with the disease. Through the regional mapping of these markers and the fortunate identification of two NF-1

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patients with a translocation in the critical region (17q11.2), a portion of the NF-1 gene itself has now been identified and its DNA sequence determined. It appears to be a large gene with an ubiquitously expressed transcript. A small number of patients have been evaluated to date for the molecular genetic defect associated with their disease phenotype. Of these, three have deletions (11, 40, and 190 kb), two have translocations (t(17;22) and t (1;17)) and two have defined single base substitutions (one mutant allele contains a T→C transition changing a leucine to proline and another has a C→T transition changing an arginine to a stop codon). Such results follow the suggestion based on the high mutation rate associated with this disease that NF-1 is a genetically heterogeneous disease and a large number of mutational events may account for this disease in different patients and families. It is interesting to note that most of the new mutations at this locus (estimated rate 1 x 10⁻⁶) that may account for up to 40% of the NF-1 cases appear to be of paternal origin. Given the high mutation rate, size of the gene, and well recognised phenotypic heterogeneity, it is imperative that the use of cosegregating linked DNA markers remains an effective means of diagnostic testing in a large number of cases. Even when the complete sequence for the NF-1 gene has been identified, it may not be possible to apply direct detection of the molecular defect on a routine basis in aspects of diagnosis and prognosis. Direct detection of mutation(s) may be feasible for some families carrying a (still unknown) predominant mutation (if present) for this disease. It is equally likely that there may not be a predominant mutation associated with this disease and that all families represent unique mutational events. Further, the genetic heterogeneity associated with this disorder may not be restricted to the NF-1 locus alone, if it follows the well accepted two hit model of tumour formation involving other genetic determinants.

The restriction fragment length polymorphism (RFLP) data on familial NF-1 reported to date suggest that although a number of combinations of restriction enzymes and NF-1 linked DNA probes yield informative polymorphisms in most populations, the index of polymorphism is variable. For example, the heterozygosity rate for MspI and c11-2B (one of the linked DNA probes) is reported to be 40% by O'Connell et al and only 9% by Ward et al. Such population specific results are common for other molecular markers as well. It may be emphasised that the usefulness of a linked genetic/molecular marker, or combination of such markers, depends on the degree of heterozygosity for each of the polymorphic sites and their index of polymorphism.

In this article, we present an analysis of this disease in the ethnically diverse population of south-western Ontario, Canada. It includes an overall impression of the impact of this disease in this population as well as a detailed clinical and molecular analysis on seven selected families. The molecular data are based on five linked probes and identify the most informative probe-enzyme combination(s) for aspects of genetic diagnosis and prediction in this population. These results are discussed in the light of other population studies of NF-1.

**Methods**

This study was part of a continuing research project in the Division of Medical Genetics and Child Health Research Institute, Children's Hospital of Western Ontario, London, Canada. It started in 1986 with a number of index cases and families ascertained through the medical genetics clinic and relevant consultants working in south-western Ontario, a population base of approximately 1·5 million. The proposal for this study was approved by the ethics committee involving human experimentation of the University of Western Ontario. Medical records were evaluated and the diagnosis of suspected cases was reviewed and confirmed by medical examination. Most first degree relatives of cooperative families were also evaluated, some more than once, particularly in the case of young children. The criteria used for diagnosing NF-1 were based on the agreement of the NIH panel. All affected subjects were assigned a severity grade for the disease, which was based on Riccardi and Kleiner and compares well with Huson et al.

Blood samples (10 to 20 ml) were collected from all available family members (first and second degree relatives) of confirmed NF-1 patients. They were used to establish permanent cell lines and for the isolation of genomic DNA for restriction fragment analysis using five NF-1 linked DNA probes on chromosome 17. The permanent cell lines will be used in future research. We will be willing to consider any collaborative proposal from international researchers. The extraction of DNA from whole blood was performed using the guanidium hydrochloride and phenol/chloroform method modified from Jeanpierre and Sambrook et al. The DNA samples were digested with appropriate restriction enzymes and fragments separated on 0·8% or 1% agarose (Biorad Laboratories) gels. DNA fragments were then transferred to Zetaprobe nitrocellulose membrane (Biorad) using alkaline blotting and covalently bound to the membrane by UV irradiation (320 nm for 15 minutes).

**DNA PROBES**

Five NF-1 linked DNA probes were used (with a number of suitable restriction enzymes) to detect six different RFLPs. The probe-enzyme combinations...
used in this study are: pUC1041-PeuII, pEW206-MspI, pEW207-BglII and HindIll, pHHH202-RsaI, and pTH1719-BgII. Inserts of known size representing these probes were isolated electrophoretically in low melting point agarose (SeaPlaque) and 25 to 50 ng of insert were radiolabelled with $^{32}$P-dCTP using the random hexanucleotide primers labelling system (Bethesda Research Labs). Radiolabelled probes were purified through Sephadex Nick-Columns (Pharmacia) and were found to have specific activities of 0.5 to 1.0 x 10^6 cpm/µg.

**HYBRIDISATION**

Membranes were prehybridised for 30 to 90 minutes in HYB solution (1 mmol/l EDTA, 7% SDS in 0.5 mol/l Na$_2$PO$_4$, pH 7.2). The prehybridisation mixture for blots probed with pTH1719 and pHHH202 included sheared human placental DNA. Blots to be hybridised with the other three probes were prehybridised in the absence of any heterologous DNA but probes were denatured in the presence of herring sperm DNA (5 µg/ml). All probes were denatured by boiling for 15 minutes, placed on ice for 10 minutes, and then allowed to hybridise to the blots in fresh HYB solution at 65°C for 16 to 20 hours (overnight). The blots were washed (2 x 20 minutes) in 5% SDS, 1 mmol/l EDTA, 40 mmol/l Na$_2$HPO$_4$ (pH 7.2) at 65°C and wrapped in Saran wrap, placed in x ray cassettes, and exposed to Kodak X-AR autoradiograph film for 24 to 72 hours at −70°C.

**Results**

We studied a total of 82 family groups in southwestern Ontario with more than one affected family member. There was a total of 243 males (111 affected) and 253 females (131 affected). A confirmed diagnosis of NF-1 was required for inclusion of individual cases in this study. However, these cases represent only a subset of all NF-1 cases in the catchment area of the Genetics Clinic of the Children’s Hospital of Western Ontario. No attempt was made to ascertain all possible families affected with this disease in this population and families with mild symptoms of NF-1 having little effect on normal development and function are not represented in the sample. At the beginning of this project, our ascertainment criteria included the presence of two or more affected subjects in the family, so our data set includes primarily familial cases of NF-1. We found a number of families in this area with a single affected member and no known family history of this disease; these cases are not included in this report and are attributed to new mutations.

All familial cases of NF-1 on our files are compatible with an autosomal dominant mode of inheritance. All transmitting parents available for medical examination had at least some symptoms of the disease, which suggests complete penetrance. Of the 82 family groups available, 14 were selected or wished to participate in the continuing research project on the genetics of NF-1. These families included a total of 32 affected cases available for further study in different age categories. The presence of NF-1 was confirmed in four generations of five families, three generations of three families, and at least two generations of six families. On a population basis, the affected subjects inherited the mutation with equal probability from the male and female parents. However, in eight families where more than one parent transmitted the mutation, the sex of the transmitting parent was exclusively male or female and in two families the frequency of transmission by the two sexes was comparable (heterogeneity $\chi^2 = 23.00$, p = 0.03). It was possible to apply birth order analysis to 18 families with 41 sibships where complete information was available. This analysis suggested that there was no significant difference between random and biologically determined birth order for affected members of the sibships. Similarly, there was no significant difference in maternal or paternal ages between affected and unaffected members of the sibships. Given the data set available, it was not possible to calculate a realistic incidence and mutation rate associated with this disease in this heterogeneous population. The frequency of this disease in this population approximates 1/3000 to 1/5000 and the mutation rate is not significantly different from the earlier reported estimates (1 x 10$^{-8}$ per gamete per generation). NF-1 is one of the most common of all the single gene diseases in this population and there is no indication of the concentration of the disease in any one of the racial groups of this heterogeneous multiracial population.

The variability of the expression of this disease is well recognised. In our sample of patients, diagnosis was made at birth in exceptional cases, a few were diagnosed during childhood, most around puberty, and still others at maturity. No biological or non-biological correlates were evident in this sample of patients to describe and explain the age of onset of the disease. Beside the age of onset, the degree of expression was among the most unpredictable variables associated with the disease. It was possible to assign a numerical score to each affected member of the 12 families where a complete medical history was available. This scoring was based on Riccardi and Kleiner to represent: minimal (1), mild (2), moderate (3), and severe (4) expression. These results were analysed with respect to the parental origin of the mutation. In our sub-sample of 65 patients (33 males
and 32 females), 29 received the mutation from the mother and 36 from the father. In this group of patients, the mean severity score for the affected parents (2.15 (SD 0.64)) was not significantly different from the mean severity score of the offspring (1.89 (SD 0.66)) and there was no association between severity of expression in the parent and the offspring. It should be pointed out, however, that of the affected subjects who received the mutation from the mother, 75% showed minimal (1) symptoms and 8.3% had moderate (3) and severe (4) manifestations. The corresponding numbers for paternally transmitted mutations was 35.7% and 35.8% respectively. This distribution is significant (p > 0.01).

The evaluation of NF-1 linked RFLPs in the subset of our population analysed so far is summarised in the table in relation to estimates of polymorphic index and in comparison to other reported populations. It shows that the six probe-enzyme combinations are polymorphic in this population with site specific variable degree of heterozygosity. Of the five probes used in this study, two are located distal to the NF-1 gene (pEW206 and pEW207), while pHHH202 and pTH1719 are proximal to this locus on the q arm of chromosome 17. In contrast, pA1041 has been localised to the p arm near the centromere. The relative position and the estimated recombination fraction for these sites in relation to the NF-1 gene are also included in the table. Probes pHHH202/pTH1719 (located proximal to the NF-1 locus) show a recombination of 10% and pEW206 shows a recombination of less than 5% distally. Thus, this combination of markers could be used with a high degree of certainty if they were found to be informative in populations and families. This table also includes genetic parameters given as observed heterozygosity and allelic frequencies which are necessary to evaluate the usefulness of individual markers. The observed heterozygosity of this population is not comparable to other reports at all sites examined. For example, the heterozygosity detected by pHHH202-RsaI is 0.54 in our sample as compared to a range of 0.25 to 0.49 reported previously. On the other hand the heterozygosity generated by pEW207-BglII in this population is comparable to other estimates (table). These population specific patterns are also reflected in the allelic frequencies, as expected. The familial segregation of these markers was further used to calculate lod scores for each of the polymorphic sites in relation to the NF-1 mutation. These estimates (not given) conform to earlier reports and suggest that the region of chromosome 17q covered by the four probes represents a total of 0.15 recombination fraction flanking the NF-1 locus.

### Discussion

Population studies on mendelian diseases are essential for evaluation of the nature and types of mutation(s) causing the disease, including explanations and hypotheses related to the frequency of the disease and a particular mutation. Such studies also allow evaluation of factors affecting severity and the impact of management or treatment protocols. Population studies are particularly relevant after the gene has been localised and cloned. The genotypic and phenotypic heterogeneity and distribution of mutation(s) across populations, an essential component in the understanding of genetic diseases, can only be obtained by population studies. The cloning of the gene for a defect does not allow 100% assurance in diagnosis and prediction, because of the nature of disease specific genetic heterogeneity; families may have unique mutations (for example, factor IX deficiency), one predominant mutation (for example, cystic fibrosis), or a single mutation in all cases (for example, sickle cell disease). Thus the cloning and localisation of a gene does not necessarily permit direct detection of the molecular defect.

### Genetic parameters for NF-1 linked DNA probes in familial neurofibromatosis type 1.

<table>
<thead>
<tr>
<th>Probe/enzyme</th>
<th>HGM</th>
<th>Heterozygosity</th>
<th>Allele frequency</th>
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<tr>
<td></td>
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<td>This report</td>
<td>Previous reports</td>
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<td>(n=51)</td>
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<td></td>
<td></td>
<td>HGM</td>
<td>This report</td>
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<tr>
<td></td>
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<td>(n=51)</td>
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<tr>
<td>pA1041/PvuII</td>
<td>D17S71</td>
<td>0.16&lt;sup&gt;0&lt;/sup&gt;</td>
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<td>pHHH202/RsaI</td>
<td>D17S33</td>
<td>0.02&lt;sup&gt;0&lt;/sup&gt;</td>
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<td></td>
<td></td>
<td>0.01&lt;sup&gt;12&lt;/sup&gt;</td>
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<tr>
<td>pTH1719/BglII</td>
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<td>0.27</td>
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<tr>
<td>pEW207/BglII</td>
<td>D17S73</td>
<td>0.04&lt;sup&gt;0&lt;/sup&gt;</td>
<td>0.32</td>
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<tr>
<td>pEW207/HindIII</td>
<td>D17S73</td>
<td>0.13&lt;sup&gt;9&lt;/sup&gt;</td>
<td>0.55</td>
</tr>
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θ = estimated recombination fraction from NF-1.
on an individual basis. This has direct implications for genetic screening and prediction.

This population study deals with genetic parameters of NF-1 in families in south-western Ontario, Canada, a genetically diverse population with much immigration. The results include familial segregation, pattern of expression, and molecular linkage. The linkage studies involve a number of closely linked DNA probes on chromosome 17. The results are informative in assessing the genetic heterogeneity of NF-1 in this population. Over the last year there have been major developments in NF-1 research including the recent discovery of the gene and part of its cDNA sequence. The predicted amino acid sequence of the resulting polypeptide appears to have been conserved throughout evolution. Although the exact function of the NF-1 gene is not known, the possibility of a role for this gene as a tumour suppressor has been suggested. The availability of the gene sequence and relevant probes allows experiments on the biochemistry and cell biology of this gene and gene product in its normal and abnormal state. These probes are informative and useful in aspects of diagnosis, prediction, and population or family specific differences and similarities.

The results reported here support a number of recognised features of NF-1, including high frequency of the disease in the population, autosomal dominant pattern of inheritance, and no effect of sex, birth order, or parental age on transmission or expression. Also, a high proportion of patients in the population have no family history (accounted for by the high mutation rate) and there is no concentration of the disease in a particular racial group or founding population. The question of parental age effect has been addressed in a number of studies. Riccardi et al. found the paternal age for NF-1 patients to be 3-15 years higher and maternal age to be 1-39 years higher than unaffected controls in a much larger sample. This may account for the predominantly higher rate of NF-1 mutation in the paternal genome as suggested by Jadavet al. Although it was not possible to estimate the incidence or prevalence with any degree of accuracy, we feel that our case load and dataset (although incomplete) compare well with other estimates. To date, there is no evidence in our sample that one sex affected with NF-1 has a lower fitness than the other and the sex of an affected subject makes little or no difference to the possibility of transmission of the mutation to the next generation. The genetic fitness of NF-1 sufferers has been suggested to be approximately half of the general population. Crowe et al. and Huson et al. have noted the reduction in fitness to be more marked in males than in females. This reduction in fitness, however, is not explained by severe intellectual handicap or disease complications causing severe morbidity or mortality before adulthood. Although Crowe et al. suggested that a significant contribution to decreased fitness in NF-1 is failure of affected subjects to marry, there is no indication of this phenomenon in our sample of this population. Although Huson et al., among others, have noted that marriages involving an affected subject have a genetic fitness of 0.80, the biological and non-biological reasons for this are not conclusive. In most of our families there is no indication of significantly increased pregnancy wastage or child mortality. We plan to continue to investigate this phenomenon with additional families and patients as they are included in our ongoing study. Our ongoing studies include the effect of transmitting parent on the severity of expression of NF-1. The data set analysed here shows that the severity score of a parent does not relate to or determine the severity score of the offspring. However, the distribution of severity score of patients receiving the mutation from the paternal or maternal sources is statistically different. Patients receiving the mutation from the mother are mostly minimally affected and only about 8% of them show moderate to severe manifestations, while the severity scores among patients receiving the mutation from the father appear to be evenly distributed. In contrast, Miller and Hall observed that the severity of expression of NF-1 was higher in patients receiving the mutation from the mother.

The DNA polymorphism data on the informative families of south-western Ontario agree with earlier reports and confirm tight linkage involving the 17q specific probes used in this study. In contrast, pA1041 (localised on 17p) in combination with PvuII (with a recombination fraction of 0.16, heterozygosity estimate of 0.16 to 0.29) was not informative in most families. Our results suggest rare recombinations involving NF-1 and pHHH202/pTH1719 as well as NF-1 and pEW206; the NF-1 gene is localised between the two sets of markers. The double crossover value involving these is expected to approach 0.5%. These restriction sites, that are closely linked to the NF-1 gene, are also highly informative in this population. They yield a high degree of polymorphism with two allelic frequencies approaching 0.5. Based on these results we recommend the use of pHHH202/pTH1719 and pEW206 as a means of flanking the NF-1 locus in any linkage analysis for detection of the presence of the NF-1 mutation with a high degree of certainty (99.5%) in informative families. With a few modifications these conclusions could be applicable to most other populations. Some of the modifications may include use of recently published NF-1 gene (exon) specific probes and primers. The reliability of these probes remains to be evaluated at the population level and is under consideration in this laboratory.
This research was financially supported by a grant from the Child Health Research Institute of Children's Hospital of Western Ontario. Valuable contributions to this research were made by Dr Hubert Soltan, Dr Victoria Siu, Dr F R Sergovich, Judy Elliott, Joanne Sutherland, Nancy Scanlan, and Ann Brown. We are grateful to Drs Ray White and David Barker for providing us with the NF-1 probes. Finally, we are grateful to the families and patients in south-western Ontario whose continued participation and cooperation is vital to the success of this ongoing research.