



# Neurofibromatosis type 1: from genotype to phenotype

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Received 16 April 2012

Revised 25 June 2012

Accepted 26 June 2012

## ABSTRACT

Although neurofibromatosis 1 (NF1) is a common Mendelian disorder with autosomal-dominant inheritance, its expression is highly variable and unpredictable. Many NF1 patients have been genotyped but few allele-phenotype correlations have been identified. NF1 genotype-phenotype correlations are difficult to identify because of the complexity of the NF1 phenotype, its strong age dependency, the relatedness of many clinical features and the huge heterogeneity of pathogenic *NF1* mutations. Some NF1 patients with a given *NF1* mutation may develop very severe disease while others with the same mutation have only mild symptoms. This phenotypic variability may be due to both modifier genes and environmental factors. Recent targeted strategies have identified several interesting candidate modifier genes.

## INTRODUCTION

Neurofibromatosis type 1 (NF1; OMIM 162200) is an autosomal disorder with a worldwide birth incidence of 1 in 2500 and a prevalence of at least 1 in 4000.<sup>1</sup> NF1 is caused by dominant loss-of-function mutations of the tumour-suppressor gene *NF1* (*Neurofibromin 1*; OMIM 613113) which encodes neurofibromin, a negative regulator of RAS proteins. The main clinical features of NF1 are multiple café-au-lait (CAL) spots, axillary freckling, Lisch nodules, optic pathway gliomas and peripheral nerve-sheath tumours.<sup>1–3</sup> NF1 patients are at an increased risk of developing both benign and malignant tumours, and NF1 is thus classified as a tumour predisposition syndrome. Although NF1 is a simply determined Mendelian disorder with complete penetrance, it is characterised by highly variable expression and marked inter- and intrafamilial variation.<sup>2</sup> Some NF1 patients with a given mutation may develop very severe disease while others with the same mutation develop a mild form. The reasons for this clinical variability are poorly understood. Evidence for the existence of modifier genes has been obtained in large familial studies.<sup>2–3</sup> Recent targeted strategies have identified several candidate modifier genes, and it is hoped that the genomics revolution will lead to further rapid progress.

## PHENOTYPIC VARIABILITY IN NF1

NF1 is a highly variable disease. It rapidly emerged that the nature of the *NF1* gene mutation was not the only source of this variation, as considerable differences in clinical expression were noted within the same family. Indeed, Carey *et al* found that three-quarters of families showed marked inter-individual differences in NF1 severity.<sup>1</sup> The

remainder of the phenotypic variability could be due to modifier genes, environmental factors or a combination of the two. The term 'modifier gene' is used here to denote any gene that influences one or several features of the NF1 phenotype. The word 'gene' is taken in its broad definition, including protein-coding sequences and microRNA and long non coding RNA genes that may modulate the NF1 phenotype. In principle, variations in the NF1 phenotype could be determined by a single modifier gene locus, or by interaction between several modifier genes. However, environmental factors might also contribute to the variable disease expression. Studies of NF1 clinical heterogeneity are hindered by the fact that the clinical course of a given patient may vary dramatically over his or her lifetime.

It is important to take other determinants into account when searching for modifier genes. For example, the average serum concentration of 25-hydroxy-vitamin D (25OHD), which plays a key role in bone metabolism and modulates the absorption of dietary calcium and phosphorus, is lower in NF1 patients than in individuals without NF1,<sup>4</sup> and the incidence of fractures has been found to be higher than in siblings and spouses without NF1.<sup>5</sup> Low 25OHD concentrations have been associated with tumours and osteopenia or fractures in adults with NF1. The serum 25OHD concentration has been found to correlate negatively with the number of dermal neurofibromas in NF1 patients.<sup>6</sup> Another study showed low 25OHD concentrations in the majority of children with NF1, potentially because of increased pigmentation and/or decreased sunlight exposure.<sup>5</sup> However, low 25OHD concentrations in children were not associated with neurofibromas, and 25OHD levels did not correlate with bone mineral density.

Age is the most important confounding factor in familial NF1 studies, many disease features being more prevalent in older patients.<sup>7</sup> Somatic mosaicism in de novo NF1 cases must also be considered, because it may lead to milder or atypical NF1 phenotypes.<sup>8–9</sup> In 2007, germline dominant loss-of-function mutations in the *SPRED1* gene were identified in patients fulfilling the US National Institutes of Health criteria for NF1, underlining a genetic heterogeneity for NF1 phenotype.<sup>10–11</sup> Legius syndrome (caused by *SPRED1* mutations) resembles a mild NF1 phenotype, with multiple CAL spots and macrocephaly, with or without axillary or inguinal freckling. By contrast, other typical features of NF1 (Lisch nodules, bone abnormalities, neurofibromas, optic pathway gliomas and malignant peripheral nerve sheath tumours (MPNST)) are lacking.

## Genotype-phenotype correlations

If the phenotypic variability of NF1 is determined primarily by modifier genes, then the phenotypic intrafamilial correlation will decrease with the degree of relatedness. However, stronger correlations between close relatives than between distant relatives could also result from shared environmental influences. This pitfall can be avoided by comparing phenotypic correlations in monozygotic (MZ) twins and other siblings.

### FIRST CLUES: NATURAL HISTORY STUDIES

Twin studies are a valuable tool for studying genetic disorders, particularly to estimate the heritability of clinical phenotypes. Heritability is defined as the proportion of phenotypic variance due to genetic variance.<sup>12–15</sup> Twins are usually considered to share the same environment, independently of their zygosity. If MZ twins have a more similar clinical phenotype than dizygotic twins, this is likely to be explained by the effect of genetic modifiers on the clinical phenotype. There are at least 30 published case reports of MZ twins with NF1. Many MZ twins have very similar clinical features (CAL spots, axillary and inguinal freckling, Lisch nodules, epilepsy, non-dysplastic scoliosis, renal vascular hypertension, unilateral ptosis and cutaneous neurofibromas).<sup>2–5, 14</sup> In principle, this is explained by identical *NF1* mutations, near-identical genomic backgrounds and very similar pre- and perinatal environments. Few MZ twins with markedly different NF1 features have been described,<sup>15</sup> but the causative mutations were not always identified. In a recent study of a pair of MZ twins, only one of whom had a NF1 phenotype, a postzygotic *NF1* gene mutation (leading to somatic mosaicism for the *NF1* mutation) was exclusively identified in the affected twin.<sup>16</sup> Plexiform neurofibromas tend to be less concordant than other features in twins with NF1. As the onset of many NF1-related tumours requires a second mutation in the wild-type *NF1* allele, the sporadic nature of such mutations has been forwarded to explain this discrepancy. Other non hereditary factors could also influence tumour initiation and growth, such as epigenetic changes, somatic mutations in other tumour-related genes and environmental factors. A recent report describes a pair of MZ twins with NF1 resulting from a de novo mutation, both of whom developed a left-sided sciatic plexiform neurofibroma that progressed to MPNST at a similar age, with pulmonary metastasis also occurring at the same age.<sup>14</sup> However, data on MZ twins, although precious, should be interpreted with care. First, the sample size is always small, with only 10 pairs in the largest series. Second, certain complications of NF1 that require imaging studies (eg, whole-body MRI for internal plexiform neurofibromas) are not always documented. Finally, many twins are studied at a young age, and their subsequent course is not known.

Clues to the existence of modifier genes in NF1 have also been provided by studies of associations between NF1 clinical features. Szudek *et al* found significant associations between Lisch nodules, optic glioma, learning disability, macrocephaly and short stature in affected parent-child pairs, but they did not adjust for the non-independence of multiple pairs of relatives from the same family or for associations between clinical features in patients.<sup>17</sup> A later analysis examined correlations between NF1 features among relatives of various degrees and confirmed that genetic factors determined the onset of particular phenotypic features in NF1.<sup>18</sup>

### GENETIC COMPONENT OF VARIABLE EXPRESSION IN NF1

Several studies of the contribution of genetic factors to the phenotypic variation of NF1 have examined NF1-related traits

in large series of multiple-case NF1 families. The patterns of variable expression are subtle, hence data on a very large number of patients and/or very large families are required.

Only three studies have assessed the inherited component of variable expression in large cohorts of well-phenotyped NF1 families.<sup>2–3, 18</sup> In 1993, Easton *et al* studied 175 NF1 patients belonging to 48 families, including six pairs of MZ twins, 76 pairs of sibs, 60 parent-offspring pairs, 54 pairs of second-degree relatives and 43 pairs of third-degree relatives.<sup>2</sup> Eight clinical features of NF1 were scored, comprising three quantitative traits (number of CAL spots, cutaneous neurofibromas, and head circumference) and five binary traits (presence or absence of plexiform neurofibromas, optic gliomas, scoliosis, epilepsy and referral for remedial education). Significant intrafamilial correlations were found for the three quantitative traits. The correlation was strongest in MZ twins, followed by first-degree relatives and then by distant relatives. The strong correlation in MZ twins suggested a major genetic component in the variable expression, while the weak correlation between distant relatives suggested that the type of mutation at the *NF1* locus itself played only a minor role. Easton *et al* concluded that the phenotypic expression of NF1 was largely determined by the genotype at modifier loci and that these modifier genes were trait-specific.

About 10 years after the study by Easton *et al*, a second large familial phenotype correlation study was published.<sup>18</sup> Szudek *et al* examined familial aggregation of NF1 features among 904 affected individuals belonging to 373 families with two or more affected members (346 families were nuclear families that included either an affected parent and one or more affected children, or two or more affected sibs). The study population was five times larger than that of Easton *et al*, and 10 clinical features were examined (CAL spots, intertrigous freckling, Lisch nodules, cutaneous neurofibromas, subcutaneous neurofibromas, plexiform neurofibromas, seizures, scoliosis, optic glioma and other neoplasms). All the phenotypic traits were treated as binary variables, and multivariate regression was used to measure associations between various classes of relatives for each feature. As Easton *et al* had previously noted, the familial patterns suggested that most of the studied clinical features had important genetic components but that their relative contribution differed according to the feature in question.

These two studies thus demonstrated a strong genetic component in NF1 variability but both suffered from certain limitations. Easton *et al* examined a limited number of patients, while Szudek *et al*, although studying a larger number of patients, could not investigate many distant relatives, owing to the small number of extended families. Moreover, this latter study did not consider CAL spots and dermal neurofibromas (major manifestations of NF1) as quantitative variables but as binary traits. In a third large familial phenotype study, Sabbagh *et al* used variance components analysis, based on maximum likelihood procedures, to estimate the proportion of phenotypic variation attributable to genetic effects.<sup>5</sup> Patterns of familial correlations were examined for 12 clinical features, including five quantitative traits (numbers of small and large CAL spots and cutaneous, subcutaneous and plexiform neurofibromas) and seven binary traits. These traits were scored in 750 NF1 patients from 275 multiplex families. With the exception of malignant neoplasms, all these features showed significant familial aggregation after adjustment for age and sex, that is, a particular feature was more likely to be present in close relatives than in the NF1 general population. The patterns of familial correlation indicated a strong genetic component for

most features, with no apparent influence of the constitutional *NF1* mutation. In accordance with the findings of Szudek and Easton, several statistically significant associations between combinations of clinical features were also found, suggesting that some NF1 features may share common genetic determinants. These results indicated a possible common repertoire of genetic modifiers for some trait combinations.

By analysing phenotype correlations in well-phenotyped NF1 families, these three large studies provided further evidence that genetic modifiers contribute to the variable expression of NF1.<sup>2 3 18</sup> This has since been confirmed by studies of NF1 mouse models.

### NF1 MOUSE MODELS: CONFIRMATION OF THE EXISTENCE OF MODIFIER GENES

Mice heterozygous for an *Nf1* 'knockout' mutation (*Nf1*<sup>+/-</sup>) are viable, fertile and cancer-prone, like their human counterparts.<sup>19</sup> However, these animals do not develop some hallmark features of the human disease, including neurofibromas and MPNSTs. The lack of neurofibromas in *Nf1*<sup>+/-</sup> mice was attributed to the very low frequency of inactivation of the remaining functional *Nf1* allele in Schwann cells. The frequency of a second-hit mutation was therefore proposed to be the rate-limiting event in the onset of neurofibromas and MPNSTs in mice. Mismatch-repair genes (*MMR*) have been proposed as putative modifier genes influencing the NF1-associated tumour frequency in humans. In addition, a low frequency of neurofibromas is observed in zebrafish with knockout mutations of three major *MMR* genes (*mlh1*, *msh2*, and *msh6*).<sup>20</sup>

### MOUSE GENETIC BACKGROUND AND NF1-ASSOCIATED TUMOUR SUSCEPTIBILITY

The frequent coexistence of *NF1* and *TP53* mutations in human MPNSTs suggested that these mutations could cooperate to promote MPNSTs. *Nf1* and *Trp53* are close together on the mouse chromosome 11. Mating of *Nf1*<sup>+/-</sup> and *Trp53*<sup>+/-</sup> mice resulted in *Nf1*<sup>+/-</sup>;*Trp53*<sup>+/-</sup>*trans* mice that exhibited increased susceptibility to MPNSTs.<sup>21</sup> This susceptibility was further increased in *Nf1*<sup>+/-</sup>;*Trp53*<sup>+/-</sup>*cis* mice carrying the two mutant alleles on the same chromatid.<sup>21</sup> Moreover, *Nf1*<sup>+/-</sup>;*Trp53*<sup>+/-</sup>*cis* mice spontaneously develop cancers associated with the human NF1 syndrome, including astrocytomas.<sup>22 23</sup> This was consistent with the observation that the *Nf1* and *Trp53* wild-type alleles could both be lost in a single genetic event in *Nf1*<sup>+/-</sup>;*Trp53*<sup>+/-</sup>*cis* mice. These results confirmed that homozygous mutations in the *NF1* and *TP53* tumour-suppressor genes cooperate in the development of MPNSTs. Moreover, tumour susceptibility appeared to be dependent on the genetic background of mice carrying the *Nf1* and *Trp53* *cis* mutations.<sup>22 23</sup> It was therefore postulated that tumour susceptibility might be influenced by *Nf1* expression levels. Hawes *et al* examined levels of *Nf1* gene expression in mouse strains with different degrees of tumour susceptibility.<sup>24</sup> They found that the strain background had as much an effect on *Nf1* expression levels as did mutation of one *Nf1* allele, indicating that animal studies of haploinsufficiency must be interpreted carefully with respect to the strain background. Because *Nf1* expression levels did not correlate perfectly with strain susceptibility to tumours, it was suggested either that variations in *Nf1* expression levels were not responsible for the differences in astrocytoma susceptibility in *Nf1*<sup>+/-</sup>;*Trp53*<sup>+/-</sup>*cis* mice, or that certain mouse strains had evolved mechanisms to compensate for differences in *Nf1* expression. Interestingly, one of the strongest determinants of astrocytoma and MPNST tumour susceptibility was

inheritance of the *Nf1*;*Trp53* mutant chromosome from the mother or father.<sup>23 25</sup> It has been postulated that an imprinted gene on chromosome 11 may be responsible for these differences in susceptibility. However, no modifier gene responsible for variations in tumour susceptibility in *Nf1*;*Trp53* *cis* mice were identified in a recent study using real-time PCR to test many of the imprinted candidate genes on mouse chromosome 11.<sup>26</sup> However, mapping of modifiers in *Nf1*<sup>+/-</sup>;*Trp53*<sup>+/-</sup>*cis* mice has led to the identification of several loci, unlinked to chromosome 11, that are responsible for susceptibility to MPNST and astrocytomas in backcross populations.<sup>25</sup> Two *nerve sheath tumour resistance* (*Nstr*) loci were identified: one near the centromere of chromosome 19 (*Nstr1*), and one at the proximal end of chromosome 15 (*Nstr2*).<sup>25</sup> *Nstr1* is syntenic with human chromosome 11q13–12, a region involved in translocations found in human MPNSTs. *Nstr2* is syntenic with human chromosome regions 5p13–15 and 8q22–24. Human chromosome region 8q22–23 is often amplified in MPNSTs and is also subject to translocation. *Nstr1* and *Nstr2* may act epistatically, with inheritance of the mutant chromosome 11 from the mother or father. Further work is required to confirm this strain-background effect, and the mechanisms underlying the effect of modifier genes on the tumour spectrum.

Mouse models do not currently mimic the full human NF1 phenotype. For example, mice lacking the alternatively spliced *Nf1* exon 23a show specific learning impairments.<sup>27</sup> In humans, exon 23a is predominantly included in most tissues and specifically skipped in central nervous system neurons. It is possible that alteration of alternative splicing could have a role as a genetic modifier of the learning disabilities in NF1 patients. A human-centered genetic approach will be necessary to identify NF1 modifier genes.

### GENOTYPE-PHENOTYPE CORRELATIONS IN NF1

Phenotypic studies of large cohorts suggest that the type of mutation in the *NF1* gene does not account for the observed phenotype variability,<sup>2 3 18</sup> and this was confirmed by genotype-phenotype correlation studies.

In addition to modifier genes, allelic heterogeneity of constitutional *NF1* mutations could also contribute to disease variability. Almost half of all NF1 cases result from sporadic mutations, and a huge number of *NF1* pathogenic mutations have been reported, hindering genotype-phenotype correlation studies. Between 5% and 10% of pathogenic mutations are large 17q11 deletions encompassing the entire *NF1* locus and neighbouring genes. Since their initial description in 1992, *NF1* deletions have been linked to a more severe clinical phenotype than intragenic *NF1* mutations. This 'contiguous gene syndrome' appears to include dysmorphic features, learning disabilities, cardiovascular malformations, childhood overgrowth, a higher burden of cutaneous neurofibromas, earlier onset of benign neurofibromas and, probably, a higher incidence of MPNSTs.<sup>28–30</sup> Some authors have speculated that increased malignancy may be explained by variations in the expression of tumour suppressor genes located in co-deleted regions.<sup>31 32</sup>

In patients with intragenic *NF1* mutations (>90% of cases), no clear-cut allele-phenotype correlations have so far been established, with the exception of a 3-bp inframe deletion (c.2970–2972 delAAT) in exon 17, which has been linked to a particular clinical phenotype characterised by the absence of cutaneous neurofibromas.<sup>33</sup> Other studies have attempted to identify genotype-phenotype correlations in patients with atypical NF1 phenotypes and/or mutation types. Patients with multiple spinal tumours but few or no other clinical features of



## Genotype-phenotype correlations

NF1 have been described, forming a subgroup or distinct genetic form of NF1 called spinal neurofibromatosis.<sup>34–36</sup> Several studies have shown that patients with spinal tumours can have various NF1 symptoms and *NF1* mutations. A recent publication reported a trend towards clustering of pathogenic changes in the 5' tertile of the *NF1* gene in patients with optic pathway gliomas.<sup>37</sup>

### INVOLVEMENT OF UNLINKED MODIFIER GENES IN PHENOTYPIC VARIABILITY

The role of the normal (wild-type) *NF1* allele (in *trans* to the primary mutation) in the variable expression of NF1 was recently investigated in a family-based association study.<sup>3</sup> Nine tag single-nucleotide polymorphisms (SNPs) in *NF1* were genotyped in 1132 individuals from 313 NF1 families. No significant deviations of transmission of any of the *NF1* variants to affected offspring was found for any of the 12 clinical features examined, based on single marker or haplotype analysis. This study provided evidence for a strong genetic component in most NF1 clinical features but no apparent influence of the *NF1* gene: neither the constitutional *NF1* mutation nor the normal *NF1* allele seemed to contribute significantly to the overall phenotypic variation of each trait. However, alterations in expression levels of the wild-type *NF1* allele were not excluded by this study. Recent reports concerning the functional structure of the human genome show that differences in transcription may also explain disease variability, and that the transcription domain of a given gene might extend very far beyond the usual regulatory sequences. This is in keeping with the different levels of *Nf1* expression observed in mouse strain backgrounds with specific phenotypes.<sup>22</sup> No determinants of *NF1* transcript levels, which could be regarded as NF1 modifier genes, have yet been found.

### BIOLOGY-DRIVEN CANDIDATE GENE STRATEGY TO IDENTIFY MODIFIER GENES

Strategies used to show the role of genetic factors in phenotypic expression can be classified into two categories: (i) a systematic approach in which the whole genome is scanned, and (ii) an approach focusing on candidate genes or pathways.<sup>38</sup> The candidate-gene approach can be defined as the study of genetic determinants of a complex trait based on: (i) generating hypotheses and identifying candidate genes that might have a pathogenic role; (ii) identifying variants (SNPs) in or near these genes; and (iii) genotyping of variants in a population, followed by statistical methods (linkage or association) to identify correlations with the phenotype.<sup>39</sup> Testing of variants in carefully selected candidates is attractive for several reasons: the number of variants is generally small, thereby avoiding severe penalties for multiple comparisons during the statistical analysis. A detailed understanding of the candidate gene product and its variants provides mechanistic insight and facilitates experimental studies to evaluate the modifier effects.

Several approaches can be used to select candidate genes. A deeper understanding of the biochemical functions of neurofibromin may lead to the discovery of interacting proteins and of upstream and downstream effectors that are critical for the development of particular phenotypic features. In both humans and mice, NF1 tumour development results from a combination of ubiquitous *NF1* heterozygosity and unpredictable *NF1* loss of heterozygosity in different cell lineages.<sup>40</sup> Neurofibroma-derived Schwann cells harbouring two mutated *NF1* alleles (*NF1*<sup>-/-</sup>) have been isolated from several neurofibromas. Mitotic recombination is the likely mechanism underlying

this loss of heterozygosity.<sup>41</sup> As mitotic recombination shows inter-individual variation, genes that control this phenomenon may partly explain the variable number of neurofibromas in NF1 patients, by influencing the somatic mutation rate.

The variable number of NF1-associated neurofibromas could also be due to variable accumulation of somatic *NF1* mutations. Two research groups have described the role of *MMR* genes in neurofibroma development in NF1.<sup>42–43</sup> Both provided evidence that a reduction in *MMR* capacity can result in *NF1* mutations in a high percentage of neurofibromas. It has been postulated that constitutional or early alterations of *MMR* genes in NF1 patients may lead to an accumulation of second hits in *NF1*, a human gene with one of the highest mutation rates. However, apart from one report, no constitutional mutations in *MMR* genes have been detected in NF1 patients.<sup>44</sup> A recent study examined whether increased tumour load in NF1 (higher number of cutaneous neurofibromas) was associated with methylation of *MMR* genes.<sup>45</sup> Titze *et al* performed methylation-specific PCR and pyrosequencing of *MMR* gene promoters most frequently involved in human cancers (*MLH1*, *MSH6*, *PMS2*, and *MSH2*) in leukocytes of NF1 patients. They found that in NF1 patients with a high number of cutaneous neurofibromas versus those with a low, methylation of two (out of six) CpG dinucleotides in *MSH2* promoter was enhanced.

Biology-driven modifier genes have also been suggested to play a role in dermal neurofibromas. Dermal neurofibromas occur in virtually all individuals with NF1. Recent elegant studies have pointed to skin-derived neural progenitors (SKPs), or their derivatives, as the cell of origin of NF1-associated dermal neurofibromas.<sup>46</sup> When *Nf1*-homozygous SKPs were autologously implanted intradermally in mice, they only gave rise to dermal neurofibromas in female mice that were pregnant at the time of implantation, and not in males or non pregnant females. This suggested that the hormonal environment may be a critical factor in the onset of dermal neurofibromas. NF1 patients typically begin to develop dermal neurofibromas around puberty, and the number and size of neurofibromas increases during pregnancy.<sup>47–48</sup> McLaughlin *et al* reported that 5% of human neurofibromas express the oestrogen receptor (ER), while 75% express the progesterone receptor.<sup>49</sup> Studies have confirmed steroid hormone receptor expression and ligand-mediated cell growth and survival in both normal human Schwann cells and neurofibroma-derived Schwann cell cultures.<sup>50</sup> Moreover, an increased potential for malignant transformation of plexiform neurofibromas has been reported during pregnancy.<sup>51</sup> The selective ER modulator tamoxifen has been tested for its ability to inhibit MPNST tumourigenesis. Tamoxifen showed potent antitumor activity in mice orthotopically xenografted with human MPNST cells, providing a rationale for clinical trials.<sup>52</sup> Thus, steroid hormones may directly influence neurofibroma initiation or progression by acting through their cognate receptors, but these effects may only apply to a subset of tumours.<sup>50</sup>

Because of their co-localisation with neurofibromin, mitochondria are also attractive NF1 modifier candidates. In *Drosophila*, neurofibromin regulates longevity and stress resistance through cAMP regulation of mitochondrial respiration and ROS production.<sup>53</sup> Recently, the role of mitochondria in tumour development has gained much attention, with reports of somatic mitochondrial DNA (mtDNA) mutations in several human cancers.<sup>54</sup> Somatic mtDNA mutations have also been described in NF1-associated neurofibromas.<sup>55</sup> Moreover, variations in mtDNA copy numbers are increasingly reported in a range of primary human cancers, suggesting that they may be

critical for cancer pathogenesis and progression.<sup>56</sup> Mitochondria contain multiple copies of circular double-stranded DNA molecules that exhibit a high degree of sequence variation across individuals. Detjen *et al* studied nucleated blood cells from four pairs of NF1 discordant MZ twins and from cutaneous neurofibromas of one twin pair, but failed to find evidence of mtDNA sequence differences or different degrees of heteroplasmy.<sup>57</sup>

## IDENTIFICATION OF CANDIDATE MODIFIER GENES IN NF1: PROOF OF CONCEPT

In a recent study, Pasmant *et al* used whole-genome high-resolution array-comparative genomic hybridisation of NF1-associated plexiform neurofibromas to identify candidate modifier genes.<sup>58</sup> For the first time, 9p21.3 deletions were identified as the only recurrent somatic alterations in these tumours. The smallest common deleted region in 9p21.3 included the *CDKN2A-CDKN2B-ARF* gene cluster and the *ANRIL* gene, a large non coding RNA. This recurrent 9p21.3 deletion was also found in a series of atypical neurofibromas (symptomatic hypercellular benign peripheral nerve sheath tumours) but not in dermal or plexiform tumours, in an independent study.<sup>59</sup> A family-based association study<sup>60</sup> was then carried out using tag SNPs located in region 9p21.3 in 1105 subjects from 306 families. Allele T of SNP rs2151280 (located in *ANRIL*) was strongly associated with a larger number of plexiform neurofibromas.<sup>58</sup> No such association was observed with dermal neurofibromas. However, the *ANRIL* SNP rs2151280 has not been tested for its possible functional effects on neurofibroma formation. To confirm the role of rs2151280, *CDKN2A*, *CDKN2B*, *ARF* and *ANRIL* expression was analysed in 124 NF1 patients' peripheral blood cells. Allele T of rs2151280 was significantly associated with reduced *ANRIL* transcript levels.<sup>58</sup> This study demonstrated the relevance of whole-genome characterisation for the identification of candidate modifier genes in plexiform neurofibromas. Targeted strategies hold great promise for the identification of novel genetic variants responsible for the heritable features and complications of NF1. However, this candidate-gene approach has been criticised for its poor reproducibility and its inability to include all possible causative genes and polymorphisms.<sup>38</sup> New genome-wide techniques may overcome these limitations.

## FROM GENETICS TO GENOMICS: PROMISE OF GENOME-WIDE ASSOCIATION STUDIES AND NEXT-GENERATION SEQUENCING IN NF1

Low-cost genotyping arrays allow researchers to perform unbiased genome-wide association studies (GWAS). GWAS can scan millions of common SNPs for their association with human complex traits.<sup>61 62</sup> The repertoire of common human DNA sequence variants now provides good coverage of all common variations of the human genome.<sup>63</sup> One strategy is to study a small number of subjects with high-density genome-wide technologies, followed by additional subjects and/or additional SNPs at regions of interest thus identified. With the advent of next generation sequencing (NGS) methods and data from the 1000 Genomes Project,<sup>64</sup> investigators must choose among (or combine) multiple strategies for creating and testing a reference panel of polymorphic sites.<sup>65</sup> Genome-wide methods hold great promise for identifying modifier genes in NF1.

## CONCLUSION

Cohort studies have shown that the clinical expression of NF1 tends to be similar in close relatives, but this similarity falls well short of that required for prognostication. The relatively minor contribution of variations at the *NF1* locus to disease expression suggests that precise knowledge of *NF1* mutations will generally be unhelpful.

The first and probably the most important step in the search for modifier genes is to select a particular clinical trait and the most relevant study population. The NF1 manifestations should be differentiated at different levels (features, consequences, and complications) in order to precisely define the relation between genetic modifiers and phenotypic characteristics.<sup>66</sup>

Modifier genes often have at least two alleles, one of which exacerbates the disease and one that suppresses it by raising the threshold for trait expression. Mimicking and perhaps enhancing the effects of naturally occurring genetic modifiers might lead to new therapeutics. A better understanding of the basis for variable disease presentation in general, and for disease suppression in particular, could improve the prediction, treatment and perhaps even the prevention of several NF1 complications.<sup>67</sup>

**Contributors** EP, MV, DV and PW wrote this review.

**Competing interests** None.

**Provenance and peer review** Not commissioned; externally peer reviewed.

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*J Med Genet* 2012 49: 483-489

doi: 10.1136/jmedgenet-2012-100978

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