A cytogenetic deletion, del(17)(q11.22q21.1), in a patient with sporadic neurofibromatosis type 1 (NF1) associated with dysmorphism and developmental delay

M Upadhyaya, S H Roberts, J Maynard, E Sorour, P W Thompson, M Vaughan, A O M Wilkie, H E Hughes

Abstract

We report the first visible cytogenetic deletion involving the NF1 gene in a patient with sporadic neurofibromatosis, dysmorphic features, and marked developmental delay. The combined evidence of molecular and cytogenetic techniques based on dosage reduction, hemizygosity for microsatellite markers, high resolution G banding, and FISH analysis, predicts this deletion to be approximately 7 Mb in size. Our findings highlight the importance of conducting a detailed cytogenetic and FISH analysis in patients with NF1 who have additional dysmorphic features or particularly severe learning difficulties.

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Neurofibromatosis type 1 is an autosomal dominant disorder, characterised by marked variation in expression, affecting approximately 1 in 3000 people.¹ The main clinical features of the disease are café au lait spots, cutaneous neurofibromas, and hamartomas of the iris (Lisch nodules). Additional complications associated with the disease include central nervous system tumours, scoliosis, plexiform neurofibromas, learning difficulties, and epilepsy.²

The gene for NF1 has been mapped to 17q11.2³⁻⁶ and was independently isolated by position cloning.⁷⁻⁹ The gene spans approximately 350 kb of genomic DNA with an open reading frame of 8454 nucleotides. The NF1 transcript is approximately 12 to 13 kb in size and is ubiquitously expressed, although at varying levels, but the predominant tissues affected in the disorder are of neural crest origin.

Neurofibromin, the NF1 gene product, is composed of 2818 amino acids and is expressed as a 250 kDa protein in brain, spleen, kidney, testes, and thymus. Neurofibromin shows some homology to a family of GTPase activating proteins (GAPs) that downregulate a cellular proto-oncogene, p21-ras. Neurofibromin represents the first cytoplasmic tumour suppressor gene product to be characterised at the cellular level. The mutation rate of the NF1 gene,

estimated at 1×10^{-4} /gamete/generation, is approximately 10-fold higher than that reported for any other single human locus. New mutations appear to be predominantly of paternal origin^{10,11} and account for approximately 50% of the cases.

Approximately 130 separate mutations have been reported to the NF1 Genetic Analysis Consortium, of which over 90 have been published.¹² Despite the high mutation rate observed at the NF1 locus, only a few mutations have been fully characterised.¹³ The rate of mutation detection in the NF1 gene has been low over the last five years. The alterations so far reported include translocations, molecular deletions, insertions, substitutions, and non-sense mutations. This is the first report to describe a cytogenetic deletion involving the NF1 gene in a patient with neurofibromatosis associated with dysmorphism and developmental delay.

Material and methods

CLINICAL DETAILS

The patient (fig 1A-E) was born at 36 weeks' gestation to healthy, 25 year old, non-consanguineous parents with no relevant family history. The birth weight was 2910 g and OFC 33 cm. Dysmorphic features were noted at birth and physical examination at 3 months of age (fig 1A) showed a prominent maxilla, low set, posteriorly rotated ears, high forehead, narrow palpebral fissures with slight ptosis on the right, micrognathia, deep palmar and plantar creases, and undescended testes. At that time his length was 56 cm and OFC 38 cm. He also had a mild patent ductus arteriosus which was later ligated. The cranial ultrasound was normal and the IVP showed mild left pelviureteric junction obstruction and hydronephrosis. Chromosomes in both blood (investigated elsewhere) and skin were reported as normal.

The proband was followed in the genetics clinic over the next few years and, despite numerous consultations, a specific syndrome could not be identified. The possibility of Smith-Lemli-Opitz syndrome was excluded by a normal level of 7 dehydrocholesterol.¹⁴ He exhibited marked developmental delay and still cannot talk or walk unaided at the age of 6 years.

At 3 to 3½ years of age he developed seizures and was then noted to have multiple café au

Institute of Medical Genetics, University Hospital of Wales, Heath Park, Cardiff CF4 4XN, UK

M Upadhyaya
S H Roberts
J Maynard
E Sorour
P W Thompson
M Vaughan
H E Hughes

Institute of Molecular Medicine, Oxford, UK
A O M Wilkie

Correspondence to:
Dr Upadhyaya.

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Figure 1 Patient at the age of 3 months (A), 14 months (B), 21 months (C), 4 years with multiple café au lait spots (D), and 4 years with axillary freckling (E).

lait spots (<six) together with axillary and groin freckling, skin changes which appeared typical of neurofibromatosis 1 (fig 1D,E). A detailed ophthalmological examination at 4 years did not show Lisch nodules and a brain CT scan was reported as normal. His height at 4½ years was 98 cm (3%) and OFC 51 cm (50%). In view of the additional clinical findings, blood

was re-examined for any deletion or alteration involving 17q.

DNA ANALYSIS

A lymphoblastoid cell line was established from the patient by transformation of B lymphocytes with Epstein-Barr virus.¹⁵ DNA was extracted

DNA analysis of the parents and affected child with 15 extragenic and intragenic markers for NF1. The marker loci are listed in order from 17p to proximal 17q. Four marker loci are not informative. The patient is heterozygous for only the 17p marker (EW301)

Locus	Marker	Father	Mother	Son
D17S58	EW301 (17p)	1,1	2,1	1,1
D17S33	pHHH202	1,1	1,1	1, (1)
CRYB1	beta 8.2	2,1	1,1	1, (1)
D17S117	VAW212	2,1	1,1	1, (1)
D17S120	VAW215	1,1	1,1	1, (1)
EV12B	NF1c2	2,1	2,1	2, (2)
EV12A	pRC9.4	2,1	2,1	2, (2)
NF1	AE25	2,1	2,1	2, (2)
	AAAT	2,1	2,1	2, (2)
	polymorphism			
	Intron 27	2,3	1,4	1
	Intron 38	3,1	2,4	4
	Intron 39	1,1	1,1	1, (1)
D17S57	EW206	2,2	2,1	2, (2)
D17S73	EW207	1,1	2,1	1, (1)
D17S117	210M1	1,1	1,1	1, (1)

Figure 2 DNA analysis based on hemizyosity for microsatellite markers in introns 27 and 38, dosage reduction, and heterozygosity with marker EW301. The extragenic flanking markers β 8.2 and EW207 showing dosage reduction in the patient are not contained in the YAC clone A43A9 and A113D7.

from lymphocytes¹⁶ and high molecular weight DNA was prepared in agarose blocks. Southern blotting and hybridisation were carried out as previously described.¹⁷ A panel of 15 polymorphic DNA markers was used to screen for NF1 gene rearrangements (table). The probes used for Southern blot analysis included pHHH202 (D17S33), VAW212 (D17S117), VAW215 (D17S120), β 8.2 (CryB1), EW206 (D17S57), EW207 (D17S73), 210M1 (D17S117), EW301 (D17S58), and the intragenic markers NF1c2, pRC9.4, and AE25.^{16,17} Genomic DNA was amplified by polymerase chain reaction (PCR). The amplified DNA samples from the patient and his normal parents were screened with two microsatellite markers located in introns 27 and 38 (fig 2) and an *RsaI* polymorphism in intron 39.¹⁸⁻²⁰

Paternity in this family was confirmed by screening with a panel of non-chromosome 17 autosomal microsatellite markers.

RNA ANALYSIS

Total RNA was extracted from the lymphoblastoid cell lines in the presence of guanidium thiocyanate according to Chomczynski and Sacchi²¹ and RNA was reverse transcribed with

murine moloney leukaemia virus reverse transcriptase (GIBCO BRL) in the presence of random hexamers at 42°C for one hour in a reaction buffer containing 50 mmol/l Tris HCl (pH 8.3), 75 mmol/l KCl, 3 mmol/l MgCl₂, 10 mmol/l DTT, and 250 μ mol/l of each dNTP. The reverse transcribed product was subjected to two rounds of amplification using the primer set III.²²

CYTOGENETIC ANALYSIS

Peripheral blood was cultured using standard techniques to produce cytogenetic preparations suitable for high resolution analysis by means of a thymidine block and deoxycytidine release.²³ GTG banding (ISCN 1985)²⁴ was by a modification of the method of Seabright.²⁵ Photographs were taken of a number of prometaphase cells using image analysis equipment (Cytoscan).

Fluorescence in situ hybridisation (FISH) studies were carried out with the D17S29 Smith-Magenis Chromosome Region (SMCR)/RARA gene digoxigenin labelled probes (Cat No P5154-DIG, Oncor, Gaithersburg, MD 20877, USA). The non-overlapping YAC clones A113D7 (290 kb) and A43A9 (270 kb) which encompass a part of the NF1 gene and the regions 5' centromeric and 3' telomeric to the NF1 gene were also used.²⁶ The gap between the YAC clones is approximately 60 kb in size. These probes were premixed with blocking DNA in Hybrisol VII (50% formamide, 2 $\times$ SSC) according to a standard protocol (Oncor). Slides were denatured in 70% formamide/2 $\times$ SSC, pH 7 at 70°C for two minutes and hybridised overnight at 37°C in a humidified chamber with the probe mixture. The post-hybridisation washes were in 50% formamide/2 $\times$ SSC, pH 7 at 43°C for 15 minutes, followed by 2 $\times$ SSC, pH 7 at 37°C for eight minutes. Detection was achieved by successive applications of monoclonal mouse antidigoxigenin, fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse antibody, and FITC-conjugated goat antirabbit antibody (Sigma). The preparations were counterstained with propidium iodide and 4', 6-diamidino-2-phenylindole (DAPI). The slides were examined with a Zeiss Axioplan epifluorescence microscope equipped for viewing FITC, DAPI, and propidium iodide and photographed using Kodachrome 400 film.

Results

Of the 15 polymorphic markers used, four were not informative. In the remaining 11, 10 markers identified a single allele band in the patient. Analysis of microsatellites present in introns 27 and 38 indicated that the patient had failed to inherit a paternal allele (figs 2 and 3). A reduction in the level of hybridisation was also observed when the DNA from the patient was screened with cDNAs AE25, P5, B3A, and the extragenic markers β 8.2, EW206, and EW207, indicating hemizyosity at these loci (data not shown).

Figure 4 GTG banding of the normal 17 (left) and the deleted 17 (right).

Figure 3 Lack of inheritance of the paternal allele within the microsatellite marker for intron 27. Lane A, DNA from the affected boy. Lane F, DNA from the father. Lane M, DNA from the mother.

Analysis with the 17p polymorphic probe EW301 localised to 17p showed the patient was heterozygous at this locus. The combined evidence of (1) dosage reduction within or near to the NF1 gene, (2) hemizygosity for microsatellite marker within introns 27 and 38, and (3) heterozygosity at the EW301 locus which maps to 17p supports the view that this patient has developed the disease (NF1) as the result of a de novo NF1 gene deletion of paternal origin rather than uniparental disomy.

Pulsed field gel electrophoresis with NF cDNA probes did not show any evidence of altered band pattern. Clearly, DNA markers closely flanking the end point of this deletion

are required to define the extent of this deletion.

RT-PCR based analysis of RNA in our patient with previously published primer set III²² spanning nucleotides 2315 to 2815 showed the transcription of an expected 500 bp normal allele. The remaining allele has not undergone any rearrangements; Western blot analysis (J Downward, personal communication) also showed the presence of a normal size neurofibromin.

Cytogenetic investigation showed that the G banding pattern was consistent with a small interstitial deletion within the proximal part of the long arm of chromosome 17. High resolution studies indicated that the breakpoints were in sub-bands q11.22 and q21.1 (fig 4). This view was supported by the FISH results. Studies on both a lymphocyte culture and a lymphoblastoid cell line showed that the YACs A43A9 and A113D7 were deleted in the abnormal chromosome 17 (fig 5, top). The RARA gene probe maps to the chromosome 17 breakpoint in the translocation t(15;17)(q22;q12 or 21.1) found in acute promyelocytic leukaemia. The signal from this probe was present in the deleted chromosome 17, but was displaced proximally towards the centromere, whereas both SMCR probe signals were located in the normal position on the two chromosome 17 homologues (fig 5, bottom). Direct measurement of pairs of standardised, enlarged, and straightened chromosome 17 homologues from 13 G banded prometaphase cells indicated that the deletion chromosome was about 8% shorter than the normal chromosome 17. This was estimated to be equivalent to a deletion of 7.4 megabase pairs (Mb) which, based on the assumption that the total number of genes in the haploid genome is 50 to 100 000, may have resulted in the loss of between 60 and 240 genes in the proband.^{27 28}

Discussion

We have identified the first visible cytogenetic deletion involving the NF1 gene in a sporadic NF1 patient with dysmorphic features and marked developmental delay. This deletion is predicted to be approximately 7 Mb in length and may contain as many as 60 to 240 genes. The molecular end points of this deletion have not been defined. However, all the eight flanking extragenic markers used in this analysis show either hemizygosity or homozygosity in the affected boy. A reduction in the dosage was observed with the DNA marker EW206 (D17S57) and β 8.2, both of which map outside a 700 kb NF1 YAC contig²⁶ and these two flanking markers are 4 cM apart.²⁹

Published data indicate that cytogenetic rearrangements and large molecular deletions involving the entire NF1 gene and various flanking regions are rare.^{12 30} To date, only five sporadic NF1 patients with a large deletion have been reported,^{29 30} but none of these was associated with a visible cytogenetic abnormality. Our patient appears to differ from these five patients in that he has more severe developmental delay and does not have Noonan-like features. At the age of 6 years, he

Figure 5 (Top) FISH with the probe A43A9 shows a signal on the normal 17 but not on the deleted 17 (both arrowed). (Bottom) FISH with the RARA and Smith-Magenis region probes shows a proximal displacement towards the centromere of the RARA probe in the deletion chromosome 17 (→) compared with the normal chromosome 17 (⇒), whereas the Smith-Magenis region probe is in the normal position on both chromosomes 17.

has marked problems with both his speech and his mobility. His phenotype is not consistent with any recognisable syndrome and no definite diagnosis had been suggested before his development of multiple café au lait spots and axillary and inguinal freckling.

The patient in this study appears to be another sporadic NF1 case with a contiguous gene deletion syndrome,³¹ although genes tightly flanking the NF1 locus have yet to be identified. Detailed molecular analysis of the size of the deletions and location of their breakpoints in all the cases involving the deletion of the entire NF1 gene will facilitate our understanding of the role of different flanking genes in the expression of the observed developmental delay and dysmorphic phenotype.

The lack of inheritance of the paternal allele indicates that the deletion must have occurred during spermatogenesis in the father. Studies with closely linked polymorphic markers have shown that approximately 95% of all new NF1 mutations are of paternal origin.^{10,11} However, in a recent study using intragenic markers, deletions observed in four of the 15 families studied, were of maternal origin.³² For Duchenne muscular dystrophy (DMD), the vast majority of deletions arise in oogenesis, while most point mutations stem from spermatogenesis.³³ A similar finding has been reported for factor 9³⁴ and ZFX and ZFY genes.³⁵ It will be important to determine whether such a bias in origin of new mutations occurs in the NF1 gene.

Benign neurofibromas in NF1 have not shown any loss of heterozygosity or large scale rearrangements, perhaps because of polyclonality. The molecular study of neurofibromas in the patient with the entire deletion of the NF1 gene will allow easier detection and characterisation of the second hit, since one allele is already missing.

It is interesting to speculate whether visible cytogenetic deletions are actually rare in the NF1 patients or if the analysis has simply not been sufficiently detailed. In an initial analysis of a lymphocyte culture (carried out elsewhere), and on examination of the cultured skin cells from this patient, the cytogenetic deletion was not discernible but was subsequently indicated in synchronised lymphocyte cultures. Although Kayes *et al.*³⁰ have already noted that sporadic NF1 cases with dysmorphism and mental impairment are more likely to carry larger deletions of the NF1 gene, our findings highlight the importance of conducting high resolution cytogenetic analysis and FISH studies in such patients with NF1 who have additional dysmorphic features or particularly severe learning difficulties.

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