

Identification of novel deletion breakpoints bordered by segmental duplications in the *NF1* locus using high resolution array-CGH

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J Med Genet 2006;43:28–38. doi: 10.1136/jmg.2005.033795

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Revised version received
13 May 2005
Accepted for publication
16 May 2005
Published Online First
8 June 2005

Background: Segmental duplications flanking the neurofibromatosis type 1 (*NF1*) gene locus on 17q11 mediate most gene deletions in *NF1* patients. However, the large size of the gene and the complexity of the locus architecture pose difficulties in deletion analysis. We report the construction and application of the first *NF1* locus specific microarray, covering 2.24 Mb of 17q11, using a non-redundant approach for array design. The average resolution of analysis for the array is ~12 kb per measurement point with an increased average resolution of 6.4 kb for the *NF1* gene.

Methods: We performed a comprehensive array-CGH analysis of 161 *NF1* derived samples and identified heterozygous deletions of various sizes in 39 cases. The typical deletion was identified in 26 cases, whereas 13 samples showed atypical deletion profiles.

Results: The size of the atypical deletions, contained within the segment covered by the array, ranged from 6 kb to 1.6 Mb and their breakpoints could be accurately determined. Moreover, 10 atypical deletions were observed to share a common breakpoint either on the proximal or distal end of the deletion. The deletions identified by array-CGH were independently confirmed using multiplex ligation-dependent probe amplification. Bioinformatic analysis of the entire locus identified 33 segmental duplications.

Conclusions: We show that at least one of these segmental duplications, which borders the proximal breakpoint located within the *NF1* intron 1 in five atypical deletions, might represent a novel hot spot for deletions. Our array constitutes a novel and reliable tool offering significantly improved diagnostics for this common disorder.

Neurofibromatosis type 1 (*NF1*) is a common autosomal dominant disorder (MIM 162200) with an incidence of 1 in 3500 individuals. The clinical features of *NF1* include café au lait spots, axillary/inguinal freckling, multiple dermal neurofibromas, and Lisch nodules as well as plexiform neurofibromas and malignant peripheral nerve sheath tumours (MPNSTs).^{1,2} The *NF1* gene from 17q11.2 has been cloned and contains 57 translated exons distributed over approximately 350 kb. About 70% of the germline mutations in the *NF1* gene result in a truncated protein due to nonsense or frameshift mutations.^{3,4} However, 5–10% of *NF1* individuals are reported to have large 17q11 deletions, encompassing the entire *NF1* gene as well as several neighbouring genes.^{5–7}

The large size of the *NF1* gene locus that needs to be studied for deletions is a methodological obstacle in routine diagnostics of the disease. Also, the complexity of the genomic architecture of 17q11 containing the *NF1* gene^{6,8–11} poses difficulties in the analysis of deletions. Although, no clear cut genotype-phenotype correlation has been established for *NF1* patients,¹² it is known that patients with large 17q11 deletions suffer from a more severe form of the disease that includes dysmorphism, mental retardation, early onset of a large number of neurofibromas, and an increased risk for the development of MPNSTs.^{13–15} The most common type of deletion spans ~1.5 Mb of 17q11 and hence is termed the "typical deletion". In approximately half of the typical deletion cases, the breakpoints were mapped to large paralogous sequences (also called low copy repeats (LCRs) or segmental duplications) flanking the *NF1* gene.^{6,8–10,16}

There have been relatively few studies reporting atypical deletions in *NF1* patients^{8,9,16–18} and this is partially due to the lack of high throughput and high resolution methodologies of deletion detection within the entire locus affected by these mutations. We here report the construction and application of an *NF1* locus specific microarray, covering 2.24 Mb of 17q11, based on a previously described PCR based non-redundant approach for array design.¹⁹ This array allows high resolution array-CGH profiling of deletions in *NF1* samples. Moreover, we present a refined bioinformatic analysis of the entire locus for identification of novel segmental duplications that might play an important role in mediating *NF1* gene deletions.

METHODS

Patient material

In total, 161 samples were studied for deletions on the *NF1* locus specific microarray, including validation cases 806 and 986 (tables 1 and 2). These included 148 lymphocyte derived DNA samples, 12 tumour (MPNST) derived DNA samples, and one cell line ASB4, which is a somatic cell hybrid from a patient mosaic for a deletion and containing only the deleted chromosome 17. DNA was isolated from these sources using standard methods. Patient samples were studied with the

Abbreviations: ANILFR, average normalised inter-locus fluorescence ratio; LCR, low copy repeat; LCR-D, LCR-distal; LCR-P, LCR-proximal; MLPA, multiplex ligation-dependent probe amplification; MPNST, malignant peripheral nerve sheath tumours; *NF1*, neurofibromatosis type 1; NPA, normalised peak area; UTR, untranslated region

approval of the local Research Ethics Committee, Faculty of Medicine, Uppsala University.

Bioinformatic analysis

The 2.24 Mb genomic sequence, encompassing the *NF1* gene, was derived from the chromosome 17 contig NT_010799.14 (human genome build 35.1) from 25587134 to 27827134 bp. As we used the sequence available from GenBank and Ensembl, we also used the same annotations for all exons of the *NF1* gene numbering them consecutively 1 to 57. The GenBank accession number for sequence retrieval of *NF1* gene exons was NM_000267. A comparison of the *NF1* exons nomenclature according to NCBI and the Neurofibromatosis (NF) Consortium is given in supplementary table 1 available at <http://www.jmedgenet.com/supplemental>. The Sequence Allocator program (abbreviated as Allocator) combines and automates three bioinformatic protocols: (i) RepeatMasker (<http://www.repeatmasker.org>) for identifying common repeats; (ii) BlastN at the NCBI server (<http://www.ncbi.nlm.nih.gov/blast>) to detect other similar sequences, with a threshold of greater than 80% identity over 50 bp, in the human genome; and (iii) Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) software for designing primer pairs in the unique genomic sequences.

Design of *NF1* diagnostic array

The array was constructed across a 2.24 Mb interval of 17q11.2 using the previously reported PCR based non-redundant approach for construction of microarrays¹⁹ which excludes all common repeats and other redundant sequences from the array. Genomic sequence of the *NF1* locus with flanking regions on chromosome 17, from 25590 to 27890 kb (according to NCBI build 35.1), was processed by Allocator. In total, oligonucleotide primers for amplification of 548 PCR products ranging in size from 200 to 998 bp (average 637 bp) were selected. Additionally, 16 control genomic loci from chromosome X and 23 autosomal control loci derived from non-chromosome 17 autosomes were treated by Allocator analogously and incorporated into the array set up.

Preparation and pooling of PCR products

Initially, PCR amplification of all fragments was done by conventional PCR using the fragment specific upper and lower primer pairs. All upper primers included a 5' universal priming site (5'-TGACCATG-3'). This site allowed a second round PCR amplification using a universal primer with an amine anchor attached to the 5' end. The amine anchor is necessary for binding of the PCR fragments to the array slide surface. Each PCR amplification reaction was done in a 100 µl volume. The size of amplified fragments was checked by agarose gel electrophoresis. Post reaction mixtures were purified using PCR Cleanup Filter Plates, Montage PCR₉₆ (Millipore, Billerica, MA, USA). Products were eluted in 50 µl deionised water. Individual PCR products were combined to form pools of 1.5 kb size on average (see supplementary table 2 at <http://www.jmedgenet.com/supplemental>) and suspended in spotting buffer (0.25 M phosphate buffer, pH 8.5; 0.00025% sarkosyl).

Array printing, hybridisation, scanning, and image analysis

The methods used for blocking CodeLink slides, DNA labelling, hybridisation, and post-hybridisation processing are detailed in the supplementary information (available at <http://www.jmedgenet.com/supplemental>). Briefly, each PCR pool was spotted on the CodeLink aldehyde slides (GE HealthCare, Slough, UK) in triplicate as a single measurement point. The slides were blocked by sodium borohydride treatment and prehybridised (5× SSC, 0.1% SDS, 0.4% BSA;

1 h, 45°C). Sonicated test and reference DNA was labelled by random priming with Cy-3 dCTP (cat. no. PA53021, GE HealthCare) and Cy-5 dCTP (cat. no. PA55021, GE HealthCare), respectively, using the BioPrime Array CGH Genomic Labeling System with purification module (cat. no. 18095-011; Invitrogen, Carlsbad, CA). Labelled DNA was suspended in the hybridisation buffer (4× SSC, 0.1% SDS, 30% formamide) and hybridised to the array (36 h, 45°C). Post-hybridisation processing consisted of four washing steps: (i) 2× SSC, 0.1% SDS, 25% formamide; 20 min, 45°C; (ii) 1× PBS; 10 min, RT; (iii) 0.2× SSC; 15 s, RT; (iv) deionised water; 15 s, RT. The slides were dried with compressed air. Image acquisition was performed using the GenePix 4000B scanner (Axon Instruments, Union City, CA). Analysis of hybridisation intensity was carried out using the GenePixPro image analysis software (Axon Instruments). The ratio of the intensities between test DNA and reference DNA was calculated using "the ratio of means" formula. The GenePixPro software subtracts the local background from the signal intensities for each spot. The average and standard deviation of the three replicas for each PCR pool were also calculated. Clones displaying a standard deviation greater than 5% of the average between a minimum of two replica spots were discarded from further analysis (unsuccessfully scored loci). The average ratio from the autosomal controls was used in the normalisation of all average ratio values from all clones on the array in each hybridisation experiment. The average normalised inter-locus fluorescence ratio (ANILFR) values were calculated in order to assess the inter-locus variation, representing a region(s) on the array. The normalised ratio for successfully scored loci from a certain, continuous region on the array was used to calculate the ANILFR value and standard deviation.

Multiplex ligation-dependent probe amplification analysis

Confirmation of array data was performed using the newly developed multiplex ligation-dependent probe amplification (MLPA) technique²⁰ with the SALSA P081/082 *NF1* MLPA kit (MRC Holland, Amsterdam, The Netherlands). Approximately 50 ng of genomic DNA extracted from peripheral blood lymphocytes was used for each probe mix. Hybridisation, ligation, and amplification were performed in thermocyclers with a heated lid (PCR Sprint, Thermo Electron, Milford, MA and GeneAmp PCR system 2700, Applied Biosystems, Foster City, CA) as previously described.²⁰ A 1 µl sample of the amplification product was analysed using an ABI 3100 Genetic Analyzer (Applied Biosystems) using ROX-500 (ABI, Warrington, UK) as an internal size standard. Analysis was performed with GeneMapper software 3.7.

RESULTS

The aim was to construct a high resolution genomic microarray spanning the *NF1* gene, including large genomic margins on both sides of this gene, and to apply this array in analysis of a representative cohort of NF1 derived samples. The underlying strategy for array design was the PCR based and entirely non-redundant approach previously reported for the *NF2* gene,¹⁹ including the application of improved surface chemistry (CodeLink, GE HealthCare) for spotting of the arrays.²¹

Construction of the *NF1* locus microarray

The construction and design of the *NF1* locus microarray demanded extensive bioinformatic analysis of the 17q11 locus, especially in view of previous reports indicating a complex genomic architecture as regards abundant segmental duplications (abbreviated as "segmentons").^{6, 8, 9} The aims

Table 1 Summary of typical deletions detected using the *NF1* locus microarray

Patient ID	Clinical phenotype	Previous deletion analysis	Array-CGH results	Size	Ref	Source
849	CAL, F, NF, DF, DD	1.5 Mb deletion, RFLP and PCR	Typical deletion (45p-155p)	1.44-1.58 Mb	2	Cardiff, UK
986†	CAL, F, DF, DD, LD	1.5 Mb deletion, RFLP and PCR	Typical deletion (45p-155p)	1.44-1.58 Mb	32	Cardiff, UK
3M 3819	CAL, F, LN, NF, AS	Total gene deletion, RFLP, PCR, and FISH	Typical deletion (45p-155p)	1.44-1.58 Mb		Cardiff, UK
2164	CAL, F, LN	1.5 Mb deletion, FISH and PCR	Typical deletion (45p-155p)	1.44-1.58 Mb		Cardiff, UK
2M 1656	CAL, F	1.5 Mb deletion, FISH and PCR	Typical deletion (45p-155p)	1.44-1.58 Mb		Cardiff, UK
637	CAL, F, NF, DF, LD	Multi-exonic deletion, RFLP	Typical deletion (45p-155p)	1.44-1.58 Mb	2	Cardiff, UK
954	CAL, F, NF, DD, LD	1.5 Mb deletion, RFLP and PCR	Typical deletion (45p-155p)	1.44-1.58 Mb	2	Cardiff, UK
1060	CAL, F, NF, DF, DD, LD, hypotonia in infancy, low IQ, probable OCL	1.5 Mb deletion, RFLP and PCR	Typical deletion (45p-155p)	1.44-1.58 Mb	2	Cardiff, UK
3M 1369	PNF	Total gene deletion	Typical deletion (45p-155p)	1.44-1.58 Mb		Cardiff, UK
280988	TMDP, CAL, F, mild MR, LAL, several CNFs	Deletion with FISH probes in microdeletion region*	Typical deletion (45p-155p)	1.44-1.58 Mb		Leuven, Belgium
222942	TMDP, cryptorchidism, hypospadias, DD, NFD, CNFs	Deletion with FISH probes in microdeletion region*	Typical deletion (45p-155p)	1.44-1.58 Mb		Leuven, Belgium
36425	TMDP, LHF, MDW, hemifacial PNF, CNFs, IQ: 71	Deletion with FISH probes in microdeletion region*	Typical deletion (45p-155p)	1.44-1.58 Mb		Leuven, Belgium
264507	TMDP, tall stature, LHF	Deletion with FISH probes in microdeletion region*	Typical deletion (45p-155p)	1.44-1.58 Mb		Leuven, Belgium
F87	CAL, F, LAL, DD, HJ, soft loose skin	Total gene deletion	Typical deletion (45p-155p)	1.44-1.58 Mb		Birmingham, AL, USA
F110	CAL, F, NFs, LN, puberty before age 10 years	Total gene deletion	Typical deletion (45p-155p)	1.44-1.58 Mb		Birmingham, AL, USA
F192	CAL, F, no further data	Total gene deletion	Typical deletion (45p-155p)	1.44-1.58 Mb		Birmingham, AL, USA
F249	CAL, F, DD	Total gene deletion	Typical deletion (45p-155p)	1.44-1.58 Mb		Birmingham, AL, USA
F256	CAL, F, subCNFs, mild MR	Total gene deletion	Typical deletion (45p-155p)	1.44-1.58 Mb		Birmingham, AL, USA
F320	CAL, F, no other data	Total gene deletion	Typical deletion (45p-155p)	1.44-1.58 Mb		Birmingham, AL, USA
F321	CAL, F, no NF, SOG, DD, DF	Total gene deletion	Typical deletion (45p-155p)	1.44-1.58 Mb		Birmingham, AL, USA
2292	CAL, dermal NFs, LN	NS	Typical deletion (45p-155p)	1.44-1.58 Mb		Cardiff, UK
2337	CAL, dermal NFs, LN, PNF, spinal NF	NS	Typical deletion (45p-155p)	1.44-1.58 Mb		Cardiff, UK
T127	CAL, F, NFs, MPNST	LOH identified by RFLP	Typical deletion (45p-155p)	1.44-1.58 Mb		Cardiff, UK
1429	CALs	NS	Typical deletion (45p-155p)	1.44-1.58 Mb		Uppsala, Sweden
1962	CALs, PNF on left leg, mild PD	NS	Typical deletion (45p-155p)	1.44-1.58 Mb		Uppsala, Sweden
3134	Uncountable CALs, mild PD	NS	Typical deletion (45p-155p)	1.44-1.58 Mb		Uppsala, Sweden

*No PCR junction fragment for deletion detected following the methodology described in Lopez-Correa *et al*†; †sample not blinded.

AS, aqueduct stenosis; CAL, café au lait spots; CNF, cutaneous neurofibroma; DD, developmental delay; DF, dysmorphic features; F, freckling; FISH, fluorescence in situ hybridisation; HJ, hypermobile joints; LAL, legs of asymmetric length; LD, learning disability; LHF, large hands and feet; LN, Lisch nodules; LOH, loss of heterozygosity; MDW, Madelung deformity wrist; MPNST, malignant peripheral nerve sheath tumours; MR, mental retardation; NF, neurofibromas; NFD, Noonan facial dysmorphism; NS, not studied previously; OCL, optic chiasm lesion; PD, psychomotor delay; PNF, plexiform neurofibroma; RFLP, restriction fragment length polymorphism; SOG, symptomatic optic glioma; TMDP, typical microdeletion phenotype.

of bioinformatic analysis were twofold. First, we hoped to identify the unique sequences in the 2.24 Mb sequence, thus allowing construction of a non-redundant genomic array. Second, we intended to perform a refined analysis of the locus in order to discover potential new segmentons. Both tasks were facilitated by use of our recently developed program, called Allocator, which allows automation of these bioinformatic procedures (de Bustos *et al*, manuscript in preparation; a program demo is available at <http://puffer.genpat.uu.se/>). Allocator identifies unique regions in the input sequence by separating non-redundant and redundant sequences, the latter being common repeats, segmentons, and other similar sequences (for example, close paralogs and pseudogenes). The program automatically designs primer pairs for PCR amplification in the identified unique regions. The redundant regions filtered out by Allocator were further analysed for the presence of segmentons, as described below.

Following analysis of the 2.24 Mb sequence, 941 unique sequence segments were identified by Allocator, including

232 non-redundant stretches spanning the *NF1* gene. The graphical output of Allocator, indicating the sizes of unique sequences, is shown in fig 1. Of the 941 unique segments, we selected 548 fragments for PCR amplification. The overall frequency of successful amplification was ~90% using total human DNA as template. After quality control on agarose gels, 444 genomic fragments, ranging in size from 200 to 998 bp (average 637 bp), were chosen for further construction of the array. The details of the primer pairs are provided in supplementary table 2 at <http://www.jmedgenet.com/supplemental>.

We aimed at maximum resolution and robust performance of array-CGH analysis in covering this locus, especially with regard to the strength of fluorescent signals from each feature on the array. Hence, for the majority of the loci we pooled two to six neighbouring PCR products and spotted them as single measurement points on the array. However, nine PCR products were not pooled and were printed individually. In total, we spotted 222 PCR pools (see

Table 2 Summary of atypical deletions detected using the *NF1* locus microarray

Patient ID	Clinical phenotype	Previous deletion analysis	Array-CGH results	Size	Ref	Source
806†	CAL, F, DF, DD	7 cM deletion	All data points on the array	>2.2 Mb	22	Cardiff, UK
T165	CAL, F, NFs, MPNST-pelvic metastases	LOH identified by RFLP	All data points on the array	>2.2 Mb		Cardiff, UK
282775	No NFs, uncountable CALs, NFD, PD, language disorder	FISH: deletion of <i>NF1</i> gene probes‡	80p–end of array	>1.33 Mb		Leuven, Belgium
T145	CAL, F, NFs, MPNST, PNF, epilepsy	LOH identified by RFLP	21p–155p	1.61–1.75 Mb		Cardiff, UK
ASB4*	Mild phenotype, 1 CAL, NF, LN, mosaicism for deletion in blood	One breakpoint in intron 8 of <i>JJAZ1</i> pseudogene, other breakpoint probably around position 148000 of AC007923	45p–144p	1.07 Mb		Leuven, Belgium
NF1_619	NF, facial DF, severe MR, cerebral astrocytoma	Deleted (size unknown); microsatellite markers and FISH	Two deletions (45p–121p and 138–end)	875 kb and >920 kb	16	Hamburg, Germany
F538	CAL, F, subCNFs	Intron 1 to intron 56; long range RT-PCR and MLPA	80p–113p (intron 1 to intron 56)	223 kb		Birmingham, AL, USA
119688	Classical NF1 but not TMDP, UDE, no CNFs at age 18, IQ: 77	Intron 1 to intron 56; breakpoints are known	80p–113p (intron 1 to intron 56)	223 kb	6	Leuven, Belgium
193	CAL, F, LN, NF, scoliosis, above average intelligence, NFs in the gum	90 kb deletion	88p–96p (intron 8 to intron 35)	93 kb	31	Cardiff, UK
2338	Clinical data not available	NS	80p–89p (intron 1 to intron 11)	66.4 kb		Cardiff, UK
162	CAL, F, LN, NF	50 kb deletion	80p–83p (intron 1 to intron 4)	25.8 kb		Cardiff, UK
F548	4 year old with CAL, F, mild scoliosis, coarse facies, SOG	Intron 5 to intron 8; long range RT-PCR and MLPA	88p (intron 7 to intron 8)	7.1 kb		Birmingham, AL, USA
F486	6 year old with CAL, F, subCNFs, PNF	Negative by long range RT-PCR, exon 1 deletion by MLPA	74p (5' UTR of exon 1)	6 kb		Birmingham, AL, USA

*ASB4 is a somatic cell hybrid containing monosomy 17; †sample not blinded; ‡deletion with PACs 962N3 and 984G23, but not with 409L16. Centromeric breakpoint is in 100 kb fragment proximal to the *NF1* gene and telomeric breakpoint is further than 984G23. 984G23 is located distal to the telomeric NF1REP and is 800 kb telomeric from the *NF1* gene.

CAL, café au lait spots; CNF, cutaneous neurofibroma; DD, developmental delay; DF, dysmorphic features; F, freckling; FISH, fluorescence in situ hybridisation; LOH, loss of heterozygosity; LN, Lisch nodules; MLPA, multiplex ligation-dependent probe amplification; MPNST, malignant peripheral nerve sheath tumours; MR, mental retardation; NF, neurofibromas; NFD, Noonan facial dysmorphism; NS, not studied previously; PD, psychomotor delay; PNF, plexiform neurofibroma; RFLP, restriction fragment length polymorphism; RT-PCR, real time PCR; SOG, symptomatic optic glioma; TMDP, typical microdeletion phenotype; UDE, unilateral deafness and epilepsy; UTR, untranslated region.

supplementary table 2 available at <http://www.jmedgenet.com/supplemental>) on the *NF1* locus microarray, including 183 measurement points from 17q11 (ID 1p to 183p), 16 pools from chromosome X (ID 184p to 199p), and 23 PCR pools from non-chromosome 17 autosomal control loci (ID 200p to 222p) (fig 2). The amount of unique sequence in each pool varied from 0.49 to 2.41 kb (average 1.5 kb). The genomic coverage of these pools varied from 0.49 to 245.87 kb. Hence, the average resolution of analysis for the *NF1* locus microarray is ~12 kb per measurement point. The *NF1* gene was covered using 44 pools (ID 75p to 118p; fig 2B) conferring an average resolution of 6.4 kb per measurement point.

Validation of the array

To validate the performance of the *NF1* locus microarray, we carried out three array-CGH experiments (fig 3). The hybridisation profile of DNA from a normal male (XY) versus reference DNA from normal female (XX) is shown in fig 3A. As expected, all the pools covering autosomal loci, including 17q11, showed fluorescence ratios for two DNA copies. The PCR pools covering chromosome X loci displayed normalised fluorescence ratios for one DNA copy. The following two experiments were performed using DNA derived from male NF1 patients in which the 17q11 deletions were previously characterised. Sample 806 is known to contain a 7 cM deletion on 17q11 encompassing the *NF1* gene.²² Upon hybridisation of this sample to our array, we observed that all the data points spanning chromosome 17 displayed normalised fluorescence ratios consistent with a

heterozygous deletion, that is, at the level of chromosome X controls (fig 3B). DNA from the male NF1 patient 986 was previously characterised to contain the typical ~1.5 Mb deletion.³² As seen in fig 3C, the array-CGH profile of this sample displays a heterozygous deletion between ID 45p and 155p (1.44–1.58 Mb). The breakpoints of the deletion were observed to be delimited by previously known segmentons and these results correspond well with previous studies.^{6–8–10–16} From the above three experiments, we considered the sensitivity and specificity of the *NF1* locus microarray for detection of single copy number deletions in the 17q11 locus to be positively established.

Application of the array to profile NF1 related samples

In order to perform a comprehensive analysis of the deletion spectrum in NF1 patients, we totally profiled 159 additional patient derived DNA samples. There was a bias in the selection of these samples. A large proportion of this cohort (96 samples, 60%) has previously been studied for point mutations in the *NF1* gene, and in 44 of these samples no point mutations have been detected (not shown). The majority of the samples (100/161) have not previously been studied for their deletion status in 17q11, while the remaining 61 samples have been analysed for DNA copy number changes by various methods and deletions larger than 5 kb were found in 30 cases (not shown). However, as we aimed for an unbiased approach towards scoring deletions by array-CGH, the details of previously characterised deletions were withheld from the group performing array-CGH.

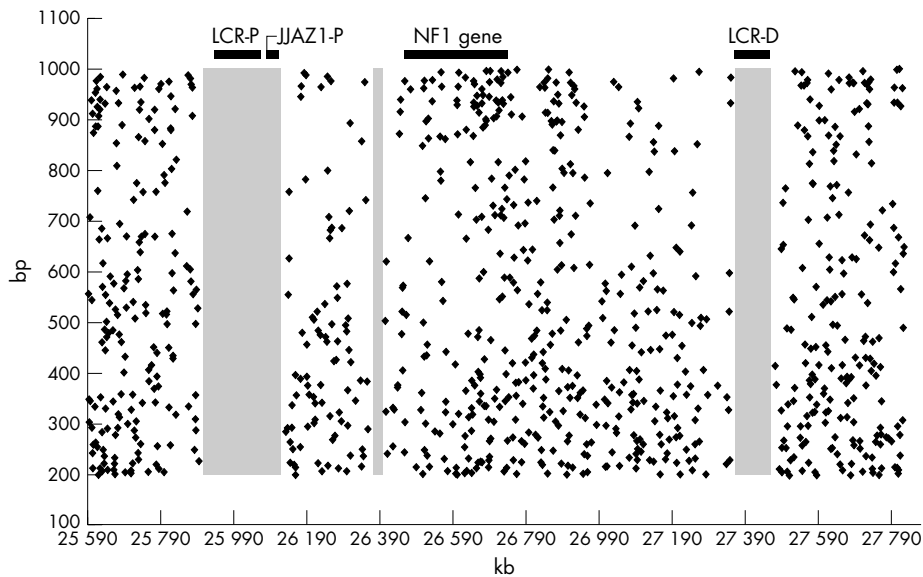


Figure 1 Graphical output from bioinformatic analysis of the 2.24 Mb sequence from chromosome 17, including the *NF1* gene, using Allocator. The approximate positions of the *NF1* gene and previously reported segments (also called LCRs), such as LCR-proximal (LCR-P), JJAZ1 pseudogene (JJAZ1-P), and LCR-distal (LCR-D), are shown as black horizontal bars.¹⁶ The shaded regions in the graph represent the large genomic segments within the studied locus, for which Allocator did not identify any unique sequences longer than 200 bp using the chosen criteria (see Methods). The x axis denotes the analysed sequence while the y axis describes the length of non-redundant sequence (200–1000 bp) identified by the program. Each point in the graph represents the length of unique sequence that could be used as a template for PCR amplification during construction of the *NF1* locus specific microarray.

Furthermore, in several instances the DNA series from our collaborators included patient samples which were already known not to contain any deletions, but these details were also not disclosed to the array-CGH investigators. In this cohort of samples, a proportion of which was previously characterised for deletions using alternative and low resolution approaches, we found heterozygous deletions of various sizes in 39 out of 161 studied cases (table 3). The observed deletions can be categorised into two groups. The first category comprises 26 cases that showed the typical 1.44–1.58 Mb deletion identified between pools 45p and 155p (fig 2A and table 1). This typical deletion is known to be mediated by segmentons known as *NF1* LCR-P and *NF1* LCR-D¹⁶ (fig 1). It is important to mention that PCR pool 45p spans the large 238 kb segmenton in the *NF1* locus and is always scored as deleted for the typical deletions (fig 2A).

The second category of deletions consists of 13 samples that showed atypical deletions, that is, heterozygous loss other than the typical 1.44–1.58 Mb (fig 2A and table 2). In two cases, namely DNA from *NF1* patient 806 and tumour (MPNST) derived sample T165, the size of the deletion was greater than the span of the microarray. Thus neither the centromeric nor the telomeric breakpoints of these aberrations could be determined. In case 282775, the proximal breakpoint of the deletion was delimited to pool 80p. The distal breakpoint, however, was beyond the coverage of the array. Therefore, in this case the deletion is at least 1.33 Mb. Case 619 is intriguing as it displays two discontinuous deletions. The first 875 kb deletion is entirely embraced by the array and encompasses the *NF1* gene, while the second one is at least 920 kb in size and extends beyond the coverage of the array towards the telomere of 17q. The latter deletion does not involve the *NF1* gene as its centromeric breakpoint (138p) is outside the *NF1* gene. In the remaining nine cases, the size of the atypical deletions ranged from 1.6 Mb to 6 kb and in each case both deletion breakpoints could be accurately determined (table 2, figs 2 and 4). An important

point to mention is that we detected the loss of one copy of genomic segments in 17q11 in three samples in which no *NF1* point mutations were previously detected. In two cases (2292 and 2337) we observed the typical 1.44–1.58 Mb deletion, whereas in sample 2338, a 66.4 kb atypical deletion, spanning intron 1 to intron 11 of the *NF1* gene, was observed.

It is also interesting to mention the results obtained from one patient among those with the typical 1.44–1.58 Mb deletions (case F110). This case was, prior to the start of this study, known to be a mosaic case for the total gene deletion. This patient was studied by FISH analysis and approximately 90% of the scored metaphases displayed a single FISH signal (Messiaen *et al*, unpublished) (data not shown). This information was, however, not known to the array-CGH team prior to their analysis of sample F110. This case was scored as a typical deletion and the levels of fluorescence ratios for deleted data points on the array-CGH profile (not shown) were not particularly different from other samples with the typical deletion. This illustrates that array-CGH is suitable for detection of aberrations using mosaic samples and confirms previous conclusions from tumour related array-CGH analyses. Indeed, the ability to accurately assess DNA copy number alterations in a mixed cell population is one of the major assets of array-CGH. A number of recent array-CGH papers have also reported the detection of DNA copy number imbalances on a heterogeneous cell background in the context of mouse tumour cell lines²⁴ and also in human primary tumours such as schwannoma, ovarian cancer, and meningioma.^{25–27} The current series of samples from *NF1* patients also included those from 12 MPNSTs, along with matching constitutional DNA available for nine of these. We detected deletions in three MPNSTs (one typical and two atypical, fig 2 and tables 1 and 2) and none of these were present in their respective constitutional DNA samples (not shown). We therefore assume that these deletions represent the second hit mutation, the first most likely being a point mutation in the *NF1* gene.

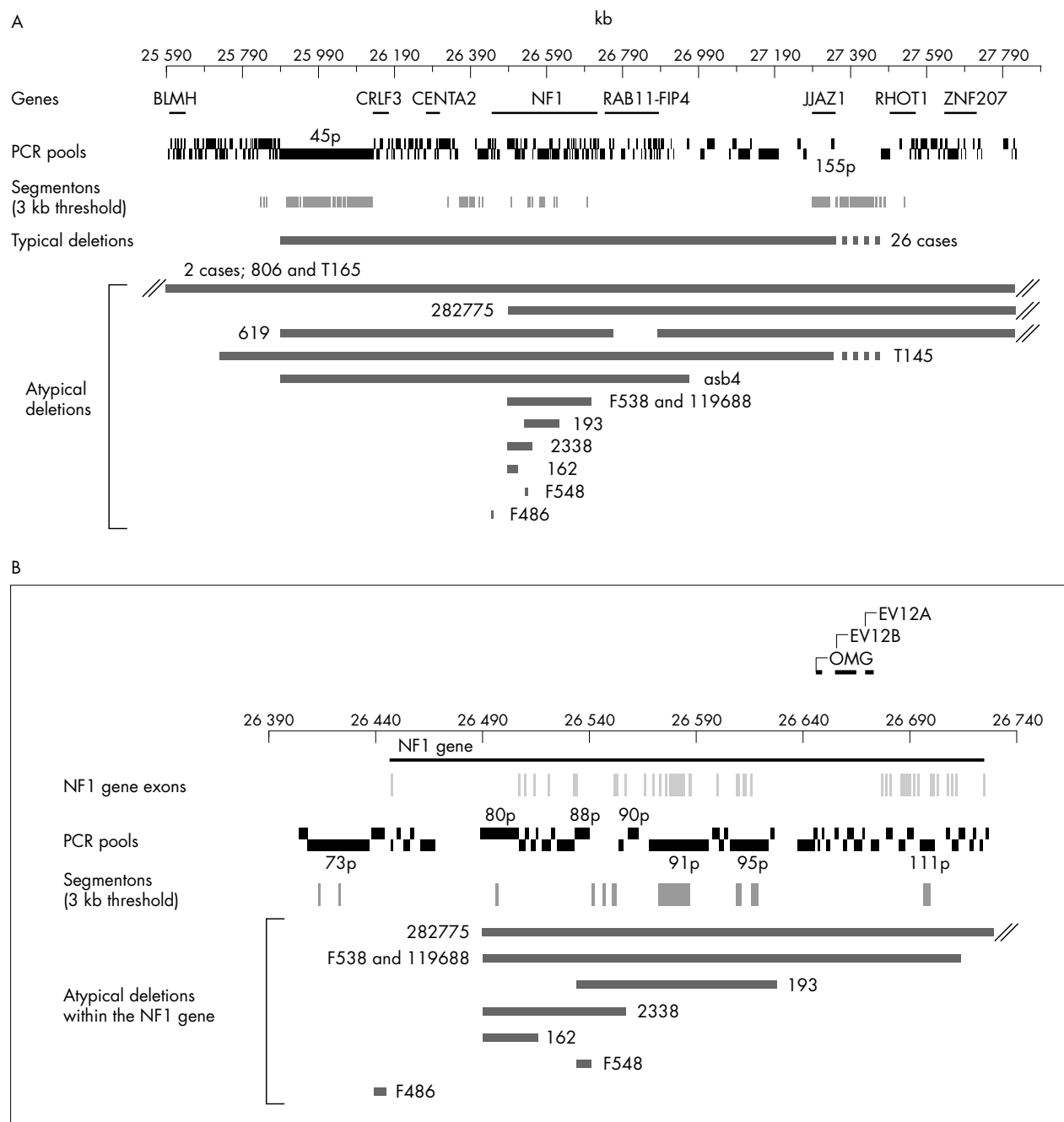


Figure 2 Map of the studied 2.24 Mb genomic segment from 17q11 and summary of all identified deletions. All elements of the figure are drawn to scale. (A) The figure displays, from top to bottom: scale of locus, known genes, PCR pools used in the construction of the array, location of segmentons, and the extent of typical as well as atypical deletions observed in NF1 derived samples. In total, 183 array measurement points were included and are shown as black rectangles in two alternating tiers. Two PCR pools (45p and 155p), which are located in the vicinity of breakpoints for the typical deletion in NF1 patients, are labelled. The light grey bars indicate the size and position of segmentons identified through bioinformatic analysis using the Allocator program using 3 kb threshold analysis for the size of segments. The dark grey bars indicate the extent of 26 typical and 13 atypical deletions that were observed by array-CGH profiling of DNA derived from 161 NF1 derived samples (tables 1 and 2). The 1.44–1.58 Mb typical deletion (25890 to 27346 kb) spans the PCR pools 45p to 155p on the array. Owing to the redundancy in the 124 kb sequence between pools 155p and 156p, we could not cover this region with any array measurement points. Hence, the distal breakpoints, in the typical deletion and the atypical deletion observed in case T145, cannot be exactly defined. This is displayed as a discontinuous bar at the end of the respective deletions. (B) Detailed view of the 350 kb locus (positions 26390 to 26740 kb) encompassing the *NF1* gene. Three genes (*OMG*, *EVI2B*, and *EVI2A*) located within the *NF1* gene intron 35 and transcribed on the reverse strand are shown. Fifty seven exons of the *NF1* gene, spanning 280.5 kb, are indicated in light grey. The black rectangles indicate the PCR pools covering the *NF1* gene. In total, 48 pools (from 72p to 118p) are shown in the figure, of which 44 pools (75p to 118p) cover the *NF1* gene, including the promoter and the 5' as well as 3' untranslated regions (UTRs). The segmentons within the locus, which were identified using a 3 kb threshold setting, are shown as medium grey bars. The dark grey bars indicate the atypical deletion profiles, in which one of the breakpoints was located in the *NF1* gene. In total, seven deletion profiles are displayed, including the 7.1 and 6 kb microdeletions in patients F548 and F486.

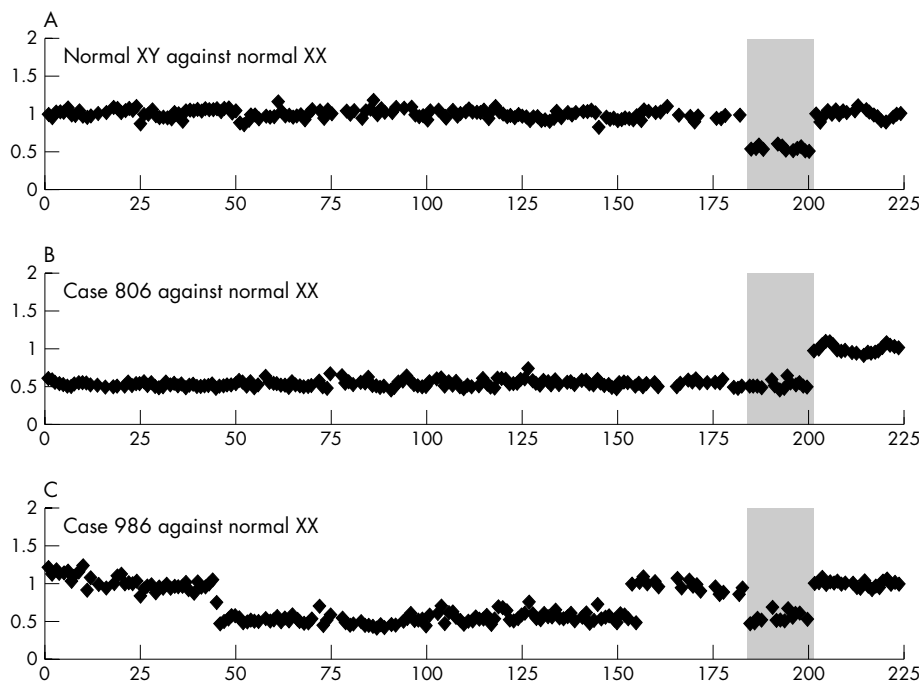


Figure 3 Validation of the *NF1* locus microarray in three experiments. The 222 array measurement points are shown on the x axis and the y axis represents the normalised, average ratio between fluorescent signals for each locus, which are derived from three independent replicate spots on the array. DNA from 10 normal female controls was pooled and used as reference DNA in all array-CGH experiments. (A) Array-CGH profile from hybridisation of DNA from a normal male (XY) versus normal female (XX). The autosomal controls displayed an ANILFR \pm standard deviation value of 0.99 ± 0.05 and the chromosome X pools provided the sensitivity to detect levels of one gene copy (0.54 ± 0.03). (B) Graph displaying the hybridisation of DNA derived from a previously characterised male patient (sample 806) and normal XX DNA to *NF1* locus microarray. As seen in the figure, the ratios of fluorescence for all 17q derived measurement points (0.53 ± 0.04) are at the level of chromosome X pools (0.52 ± 0.04) confirming the presence of a heterozygous deletion in this patient across the entire array. The non-chromosome 17 autosomal control pools display ANILFR for two gene copies (1.00 ± 0.05). (C) Array-CGH profile of hybridisation of DNA from previously characterised case 986 and normal female DNA. We identified the 1.44–1.58 Mb deletion from 45p to 155p (0.53 ± 0.07). The ANILFR values for chromosome X and autosomal controls were as expected (0.55 ± 0.06 and 1.00 ± 0.04 , respectively).

Confirmation of array-CGH findings using MLPA

We applied MLPA analysis as an independent methodology to confirm the *NF1* gene deletions detected by array-CGH, as it provides exon-level resolution in profiling the gene of interest for DNA copy number changes.²⁰ We used the commercially available SALSA P081/082 *NF1* MLPA kit (MRC Holland) for deletion detection across the *NF1* gene. In total, 81 probes are included in the kit, of which 56 (ID 1 to 56) span the *NF1* exons (except exons 7 and 9) and 25 (ID 57 to 81) cover autosomal control loci. The normalised peak area (NPA) for each probe is indicative of the relative copy number of the corresponding exon when compared to the control loci. In total, DNA from 29 *NF1* samples, including six typical deletion cases, all 13 atypical deletion cases, and 10 non-deleted cases were profiled by MLPA. For the typical deletion cases, in which the entire *NF1* gene was deleted, a heterozygous deletion of exons 1–57 was detected (data not

shown). The samples which were negative for deletions by array-CGH, were also negative for copy number alterations within the *NF1* gene by MLPA (data not shown). Upon analysis by MLPA of the 13 atypical deletion cases, we observed that there was concordance in all but one (case F548) with the data derived from array-CGH. The data from patient F548 are in partial disagreement, owing to the array design for the *NF1* gene exons deleted in this case (see Discussion below). Four representative profiles from these experiments are shown in fig 4 (panels B, D, F, and H). Figure 4B displays the detection of a 223 kb deletion encompassing *NF1* exons 2 to 56 in sample 119688 by MLPA. For comparison, the array-CGH data for the same sample (fig 4A) indicated a heterozygous deletion from intron 1 to intron 56. Similarly, the two 7.1 and 6 kb microdeletions detected by *NF1* locus array in samples F548 and F486, respectively, were also confirmed by MLPA analysis (fig 4F,H). In summary, in all the analysed cases, the data obtained from MLPA independently verified all the analysed deletions detected by array-CGH.

Table 3 Summary of previous mutation analysis and our array-CGH results

Previous mutation analysis	No.	Deletions detected by array-CGH
Previous point mutation or deletion analysis	117*	
Mutations detected	67*	33
No mutations detected	50	3
Mutation analysis not performed	44	3

*12 of these are MPNSTs.

Identification of novel segmentons within 17q11

From the above analysis of DNA dosage alterations we identified 13 atypical deletions. We also observed that 10 of these 13 deletions shared a common breakpoint either on the proximal or distal end of the deletion. In five samples (282775, F538, 119688, 2338, and 162) we identified a common proximal breakpoint within intron 1 of the *NF1* gene delimited by the data point 80p. This pool contained 1.7 kb of sequence in four PCR fragments and it spanned

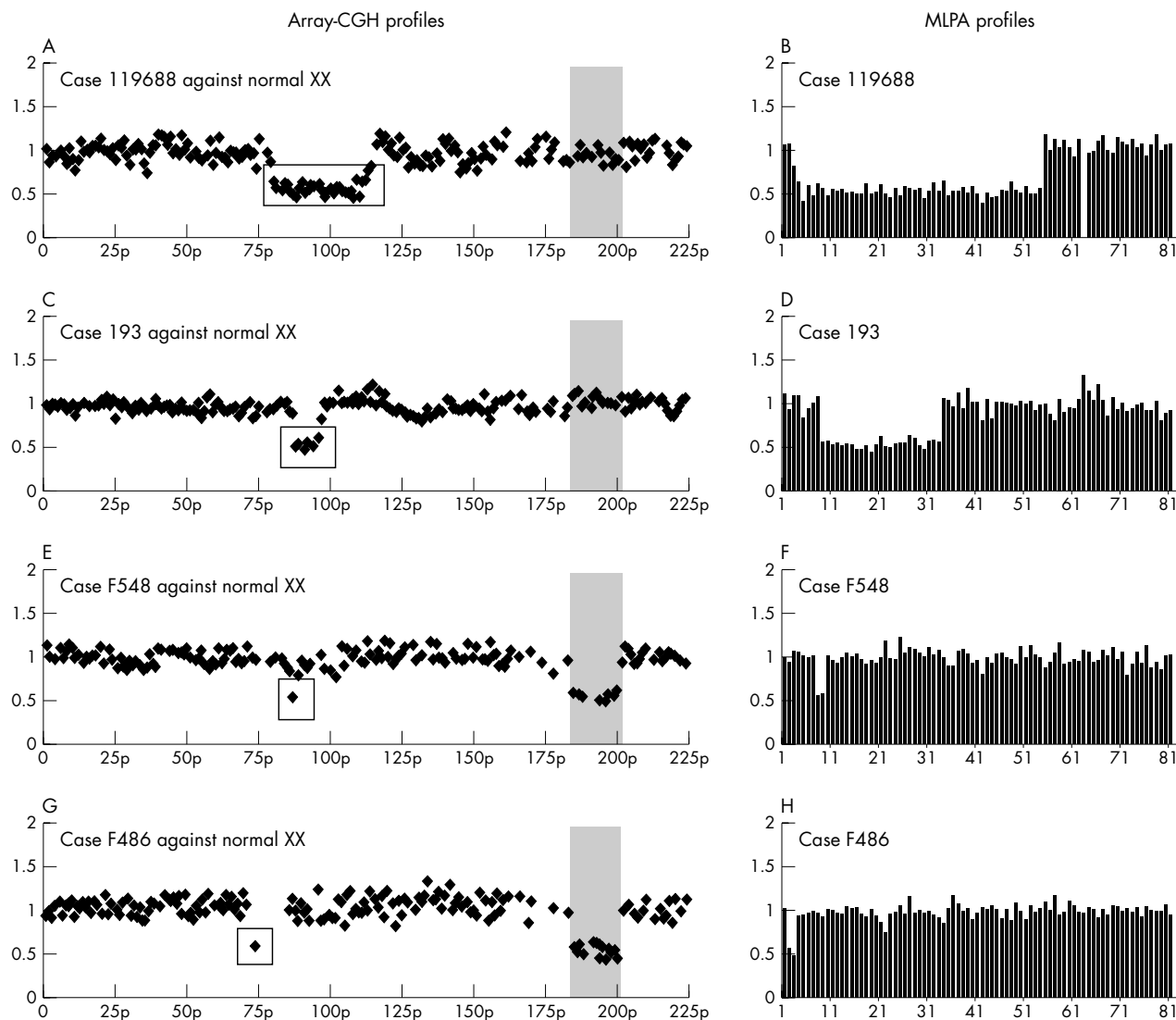


Figure 4 Detection of atypical deletions in NF1 patients using the *NF1* locus specific microarray and confirmatory MLPA analysis. The array-CGH profiles of DNA derived from four NF1 samples versus normal female reference DNA are shown in panels A, C, E, and G. The general layout of array-CGH profiles follows the structure described in fig 3. Rectangles in the panels indicate the measurement points displaying fluorescence ratios consistent with a heterozygous deletion. Panels B, D, F, and H show the corresponding MLPA analysis for each patient. The x axis displays the 81 probes (ID 1 to 81) from the MLPA SALSA kit. The normalised peak area (NPA) value of each probe (y axis) indicates the relative copy number of the corresponding exon when compared to autosomal control loci. (A) An array-CGH profile of the 223 kb heterozygous deletion (ID 80p to 113p, intron 1 to intron 56) in female NF1 patient sample 119688. The normalised fluorescence ratios for the deleted measurement points are 0.55 ± 0.05 and for autosomal controls are 1.00 ± 0.09 . An identical array-CGH profile was detected in another female NF1 sample (case F538, profile not shown, see table 2). (B) MLPA profile from analysis of case 119688. The heterozygous deletion of the *NF1* gene exons 2 to 56 is detected by 51 probes (ID 4 to 54) with an NPA value of 0.53 ± 0.05 . (C) Detection of a 93 kb heterozygous deletion in female patient 193, who has been previously characterised as having a 90 kb deletion within the *NF1* gene.²³ Array-CGH analysis of DNA from this patient displayed a heterozygous deletion (ID 88p to 96p; ANILFR 0.53 ± 0.04) spanning 93 kb of the *NF1* gene from intron 8 to intron 35. (D) Profile from MLPA analysis of case 193. The graph displays one copy loss of 25 probes (ID 9 to 33; NPA 0.52 ± 0.04) which encompass the *NF1* exons 8 to 35. (E) Detection of a small heterozygous deletion within the *NF1* gene in case F548. The loss of one DNA copy is detected between intron 7 and intron 8 by a single PCR pool (88p) with a normalised fluorescence value (0.53); the genomic coverage of this pool is 7.1 kb. (F) Confirmatory MLPA analysis of case F548. We detected a heterozygous deletion spanning *NF1* exons 6 to 8 (ID 8 to 9; NPA 0.56 ± 0.01). (G) Array-CGH profile of NF1 case F486. We detected heterozygous deletion of a single measurement point (74p; normalised ratio of fluorescence 0.60) spanning the 5' UTR of the first exon of the *NF1* gene. Empty space following pool 74p and indicating data points not scored is a result of a printing failure of the particular array used in the analysis of this patient. (H) Confirmatory MLPA analysis of case F486. Two probes (ID 2 and 3) within exon 1 of the *NF1* gene displayed NPA values (0.52 ± 0.05) consistent with one copy loss.

17.9 kb of genomic sequence (fig 2B). In two additional samples (cases 193 and F548) displaying intragenic microdeletions, we observed a common breakpoint on the proximal end of the deletions. This was encompassed in array data point 88p, which contained 1.2 kb of sequence in two PCR fragments and spanned 7.1 kb of the locus (fig 2B). We also observed that some of the atypical deletions apparently had

the same centromeric or telomeric breakpoints, when compared with the breakpoints for the typical 1.44–1.58 Mb deletion. In two samples (619 and ASB4) the centromeric breakpoint was defined in pool 45p and in case T145 (MPNST derived DNA) the telomeric breakpoint was located in pool 155p. As described before, the proximal and distal breakpoints of the typical 1.44–1.58 Mb deletion are located in the

vicinity of pools 45p and 155p, respectively. These observations have prompted us to perform a refined bioinformatic analysis across the 2.24 Mb with the aim of localising novel segmentons in the vicinity of breakpoints for atypical deletions. Identification of all types of redundant sequences is a by-product of the Allocator program. We therefore utilised this part of the output and further processed it to identify new segmentons.

Contiguous pieces of redundant sequence with a minimum size of 3 kb, identified by Allocator, were regarded as potentially containing segmental duplications. For a more detailed analysis, these regions were blasted against the reference sequence of the human genome (<http://www.ncbi.nlm.nih.gov/genome/seq/HsBlast.html>), using default parameters. Sequences showing at least 90% similarity over 1000 bp to another location in the human genome were considered to be segmental duplications. The 33 segmental duplications resulting from this analysis are shown in fig 2 and in table 4. Special attention was paid to the segmentons that were present in pools associated with the recurrent breakpoints for atypical deletions (80p and 88p) (fig 2B and table 2). Pool 80p, which was the common proximal breakpoint in five atypical deletions, displays high similarity to 4.7 kb on chromosome 13 (18569.3 to 18574 kb). Pool 88p was observed to be the common breakpoint in two atypical deletion cases. Blast analysis revealed that the genomic sequence of this pool displayed high sequence similarity to regions on chromosomes 18 and 21 of similar size, which means that there are three copies of this segmenton within the haploid genome. In summary, we identified two novel segmentons bordering proximal

breakpoints in atypical deletions; these should be studied further for their role in mediating deletions in NF1 patients.

DISCUSSION

We report a new tool for accurate, high throughput, and cost effective analysis of disease causing deletions for a very common genetic disorder. The average resolution of analysis for the whole 2.24 Mb locus is ~12 kb per measurement point. However, the average resolution for the *NF1* gene is twice as high (~6 kb per array data point), that is, 57 *NF1* gene exons are resolved by 44 measurement points, which is close to exon level resolution. Indeed, in one case we detected a deletion involving only a single exon (case F486, exon 1, fig 2). We also show that the same array allows, in a single experiment, detection and simultaneous sizing of deletions from 6 kb up to the megabase range, including deletions as large as the entire extent of the array. Furthermore, we analysed a large series of patient samples (161 in number) and observed 39 deletions affecting the *NF1* tumour suppressor gene, of which 26 were the typical 1.44–1.58 Mb deletions and 13 were atypical deletions. If three cases of somatic deletions in MPNST samples are excluded from the series of deletion cases, then we detected 25 typical and 11 atypical deletions occurring in the constitutional DNA of NF1 patients. These results are in agreement with previous studies^{6–9} reporting a high frequency of the typical deletions, which are mediated by the large segmentons flanking the *NF1* gene on both sides. Moreover, our study clearly indicates that there exists considerable heterogeneity of deletion breakpoints among patients with *NF1* gene microdeletions. One disadvantage of the current version of the *NF1* locus microarray is that with the chosen settings for the Allocator program and the criteria for the construction of the array, it is not possible to differentiate between the 1.44–1.58 Mb and 1.2 Mb deletions mediated by *NF1* LCRs (proximal and distal) and *JJAZ1* gene elements, respectively. The 237.8 kb genomic sequence, spanning *NF1* LCR-P and *JJAZ1*-P, is identified as redundant by Allocator and hence, no measurement points could be created to resolve these two regions.

No clear genotype-phenotype relationship has been found in NF1.¹² This may be either due to the influence of additional modifier loci in different individuals or the variable nature, location, and developmental timing of the somatic mutations of the *NF1* gene which determine the severity of disease. Although a correlation between the presence of large *NF1* gene deletions and clinical features (including dysmorphic features, learning disability, and developmental delay) has been described,¹³ we find that not all patients with *NF1* deletions have this specific phenotype.² In this study, we did not observe any obvious relationship between the size of *NF1* deletion and specific clinical features. This indicates that both unlinked modifying genes and the normal allele may be involved in the development of particular clinical features in NF1, and that the relative contributions may vary for different features. We also identified new segmentons bordering breakpoints of atypical deletions and these should be further studied to determine their role in causing these rearrangements. In the case of at least one of these novel segmentons (located within intron 1 of the *NF1* gene and contained within the 80p array data point) we found an association with five separate deletion breakpoints. In each of these five cases, the deletions extended towards the telomere of 17q but varied considerably in size from 25.8 kb to >1.33 Mb. However, two of the deletion profiles derived from different patients (cases 119688 and F538, fig 2) were apparently identical. Hence, it is tempting to hypothesise that the segmenton, contained within the 80p array data point, represents a new hot spot for *NF1* gene deletions, which warrants further study. It should be stressed in this context

Table 4 Segmental duplications identified in the analysed 17q11 segment using a 3 kb threshold setting for minimum redundant sequence length

Segmenton number	Start on chr. 17 sequence	End on chr. 17 sequence	Size of the segmenton
1	25840801	25841920	1119
2	25846358	25850382	4024
3	25852953	25854201	1248
4	25904240	25936929	32689
5	25940406	25941527	1121
6	25952455	26023997	71542
7	26027961	26036380	8419
8	26038649	26050367	11718
9	26053161	26061266	8105
10	26063421	26130956	67535
11	26329732	26332515	2783
12	26360373	26384016	23643
13	26387064	26402110	15046
14	26412845	26413874	1029
15	26422217	26423581	1364
16	26496020	26497571	1551
17	26541266	26542658	1392
18	26546257	26547392	1135
19	26550729	26552976	2247
20	26572600	26587209	14609
21	26608916	26611300	2384
22	26615910	26619020	3110
23	26696521	26699863	3342
24	27288579	27313290	24711
25	27315594	27337683	22089
26	27352384	27356101	3717
27	27360773	27362123	1350
28	27364177	27386904	22727
29	27389104	27452780	63676
30	27457013	27459848	2835
31	27466425	27470656	4231
32	27479299	27482802	3503
33	27530551	27531651	1100

that this segmenton is present at least twice per haploid genome and on two different autosomes (that is, chromosomes 13 and 17). Assuming that our hypothesis regarding the involvement of "segmenton 80p" is correct, this indicates an as yet uncharacterised mechanism mediating deletions within the *NF1* gene.

Our current series of NF1 derived samples included 12 samples from MPNSTs. Three of these disclosed deletions within the studied 2.24 Mb locus and these were not present in the constitutional DNA of affected patients. Interestingly, two of these deletions were entirely contained within the 2.24 Mb locus, including one sample (T127, table 1) that showed the typical deletion breakpoints. Previous studies analysing the *NF1* gene in MPNSTs have shown a varying percentage (55–100%) of loss of heterozygosity at 17q.^{28–30} A recent FISH based study of MPNSTs also identified deletions of the *NF1* gene in all eight analysed MPNST cases.³⁰ However, as far as we are aware, this is the first report disclosing the typical *NF1* locus deletion (located between the two major segmentons flanking the *NF1* gene) in an MPNST sample. The absence of large deletions in the constitutional DNA of these patients indicates that small genetic lesions, most probably point mutations, are sufficient to cause malignancy in some NF1 patients. This result also suggests that abnormal intra-chromosomal recombination, mediated by these flanking segmentons, is also causing deletions of the *NF1* gene in somatic cells, via an as yet unknown mechanism that likely occurs during mitosis.

In only one case (F548) were the data obtained by array-CGH not in complete agreement with our MLPA analysis (fig 4E,F) and previous data. The deletion in F548 was previously mapped from exon 6 to exon 8 using long range PCR and MLPA and was confirmed independently by our MLPA analysis. However, array-CGH analysis detected the deletion starting in intron 7 and ending in intron 8. This discordance between previous data and our array-CGH data can be explained by the design of the *NF1* locus microarray. Pool 87p spans a 8.5 kb genomic sequence between intron 5 and intron 7. Most of the sequence (7.75 kb out of 8.5 kb; 92%) covered by pool 87p is within intron 5. Pool 88p also consisted of two PCR products and spans a 7.1 kb genomic sequence between intron 7 and intron 8. The deletion in F548, which spans intron 5 to intron 8, was detected by pool 88p but not by 87p. This can be explained by assuming that the proximal breakpoint of the deletion in F548 is situated closer to exon 6 than exon 5 and consequently only a small fraction of intron 5 is deleted. This would result in a situation where most of pool 87p is not deleted and hence the fluorescence ratio for this measurement point was consistent with two copies.

The array-CGH methodology has emerged as a revolutionary and versatile platform over the past few years, allowing a number of different assays to be performed.³¹ Rapid development of this technology is likely to continue over the next few years, with improvements in coverage and resolution as the main focus. This will have a major impact on genetic research and the routine diagnostics of genetic disorders. From our *NF1* locus array-CGH data, we show that the shortest stretch of unique sequence which allows reliable detection of single copy number changes is 490 bp, using fluorescently labelled total human DNA in the hybridisation experiments. In conclusion, even at the current resolution, our array constitutes a novel and reliable tool offering significantly improved diagnostics for this common disorder.

ACKNOWLEDGEMENTS

We would like to thank Birgitta Sinder Wilen and Anne-Marie Westlund for excellent technical assistance.

ELECTRONIC-DATABASE INFORMATION



The supplementary tables are available at <http://www.jmedgenet.com/supplemental>. Other URLs mentioned in this study are as follows: RepeatMasker, <http://www.repeatmasker.org>; BLAST, <http://www.ncbi.nlm.nih.gov/blast>; Primer3, <http://frodo.wi.mit.edu/cgi-bin/primer3/primer3 WWW.cgi>; and Human Blast Sequences, <http://www.ncbi.nlm.nih.gov/genome/seq/HsBlast.html>.

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This work was supported by grants from the U.S. Army Medical Research and Materiel Command, award no. W81XWH-04-1-0269, the Swedish Cancer Foundation, Wallenberg Consortium North, The Swedish Research Council, and Uppsala University to JPD, and the Danish Platform for Integrated Biology of the Danish Basic Research Fund to LB

Competing interests: none declared

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