Open access • Journal Article • DOI:10.1002/HUMU.21271

NF1 microdeletions in neurofibromatosis type 1: from genotype to phenotype†

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Published on: 01 Jun 2010 - Human Mutation (John Wiley & Sons)

Topics: Contiguous gene syndrome and Neurofibromatosis

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Human Mutation

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Journal:	*Human Mutation*
Manuscript ID:	humu-2010-0043.R1
Wiley - Manuscript type:	Mutation in Brief
Date Submitted by the Author:	02-Apr-2010

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HUMAN MUTATION Mutation in Brief #____ (2010) Online

MUTATION IN BRIEF

HUMAN MUTATION

Received 27 January 2010; accepted revised manuscript 12 April 2010.

HUMAN MUTATION Mutation in Brief #____ (2010) Online

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NF1 Microdeletions in Neurofibromatosis Type 1: From Genotype to Phenotype

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Communicated by Dominque Stoppa-Lyonnet

ABSTRACT: In 5-10% of patients, neurofibromatosis type 1 (NF1) results from microdeletions that encompass the entire *NF1* gene and a variable number of flanking genes. Two recurrent microdeletion types are found in most cases, with microdeletion breakpoints located in paralogous regions flanking *NF1* (proximal *NF1-REP-a* and distal *NF1-REP–c* for the 1.4 Mb type-1 microdeletion, and *SUZ12* and *SUZ12P* for the 1.2 Mb type-2 microdeletion). A more severe phenotype is usually associated with *NF1* microdeletion patients than in those with intragenic mutations. We characterized *NF1* microdeletions in 70 unrelated *NF1* microdeleted patients using a high-resolution *NF1* custom array comparative genomic hybridization (CGH). Genotype-phenotype correlations were studied in 58 of these microdeletion patients and compared to 389 patients with intragenic truncating *NF1* mutations and phenotyped in the same standardized way. Our results confirmed in an unbiased manner the existence of a contiguous gene syndrome with a significantly higher incidence of learning disabilities and facial dysmorphism in microdeleted

Received 27 January 2010; accepted revised manuscript 12 April 2010.

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patients compared to patients with intragenic *NF1* mutations. Microdeleted NF1 patients also showed a trend toward significance for childhood overgrowth. High-resolution array-CGH identified a new recurrent ~1.0 Mb microdeletion type, designated as type-3, with breakpoints in the paralogous regions middle *NF1-REP-b* and distal *NF1-REP–c*. ©2010 Wiley-Liss, Inc.

KEY WORDS: Genotype-phenotype correlation; High-resolution custom CGH; Neurofibromatosis type 1; *NF1* locus microdeletion; Standardized phenotype questionnaire; Type-3 *NF1* microdeletion

INTRODUCTION

Neurofibromatosis type 1 (NF1; MIM# 162200) is an autosomal disorder with an estimated incidence of 1 in 3500 live births (Carey et al., 1986). NF1 is due to autosomal dominant loss-of-function mutations of the *NF1* gene (neurofibromin 1; NM_000267), a tumour suppressor gene located at 17q11.2 and containing 60 translated exons distributed over ~300 kb. In 5-10% of patients, NF1 is caused by genomic microdeletions that encompass the entire *NF1* gene and a variable number of immediately flanking genes (Kluwe et al., 2004). The majority of such NF1 patients have one of two recurrent types: type-1 and type-2 microdeletions. The typical type-1 microdeletion is 1.4 Mb long, contains *NF1* and at least 14 additional protein-coding genes, four pseudogenes and two microRNAs (Supp. Figure S1) and is caused by non-allelic homologous recombination (NAHR) between paralogous sequences flanking the *NF1* gene: the *NF1* proximal and distal low-copy repeats (LCRs): *NF1-REP-a* and *NF1-REP-c* (Dorchner et al., 2000; López Correa et al., 2000; Jenne et al., 2001, 2003; Forbes et al., 2004; Raedt et al., 2006). The typical type-2 microdeletion is smaller (1.2 Mb) and has breakpoints located in the *SUZ12* gene (suppressor of zeste 12 homolog; NM_015355) and its pseudogene *SUZ12P* (Petek et al., 2003; Kehrer-Sawatzki et al., 2004; Steinmann et al., 2007; Roehl et al., 2010). Even less frequent, atypical *NF1* microdeletions with non-recurring breakpoints have also been reported (Riva et al., 2000; Kehrer-Sawatzki et al., 2003, 2008; Venturin et al., 2004a; Mantripragada et al., 2006; Pasmant et al., 2008).

The main features of NF1 include multiple café-au-lait (CAL) spots, skin-fold freckling, Lisch nodules, and benign peripheral nerve sheath tumours (dermal and plexiform neurofibromas) whose occurrence, number, and size vary greatly from one patient to another, even from within the same NF1 family (Friedman, 1999). More than half of NF1 patients will also develop plexiform neurofibromas, of which about 5-10% will progress into a malignant peripheral nerve sheath tumour (MPNST) (Evans et al., 2002).

While NF1 represents a simply determined Mendelian disorder with complete penetrance, it is however characterized by highly variable expressivity in both the number of major features and the occurrence of complications (Friedman, 1999; Sabbagh et al., 2009). A more severe clinical phenotype has been reported in NF1 patients carrying genomic microdeletions that involve the entire *NF1* gene, compare to patients with intragenic *NF1* mutations. This contiguous gene syndrome does appear to include dysmorphic features (Upadhyaya et al., 1998; López Correa et al., 1999, 2000; Streubel et al., 1999; Riva et al., 2000; Castle et al., 2003; Venturin et al., 2004b), learning disabilities (Tonsgard et al., 1997; Upadhyaya et al., 1998; Korf et al., 1999; López Correa et al., 1999, 2000; Streubel et al., 1999; Riva et al., 2000; Castle et al., 2003; Descheemaeker et al., 2004; Venturin et al., 2004b), cardiovascular malformations (Riva et al., 2000; Venturin et al., 2004b, 2005; Mensink et al., 2006), childhood overgrowth (Spiegel et al., 2005), a higher tumour burden and earlier onset of benign neurofibromas (Leppig et al., 1997; López Correa et al., 2000; Castle et al., 2003), and probably, a higher incidence of MPNSTs (De Raedt et al., 2003) and other malignancies (López Correa et al., 2000). While many reports of *NF1* microdeleted patients (currently > 150 cases) (Mensink et al., 2006) have been published since the initial study in 1992 (Kayes et al., 1992), to date, little is known about this genotype-phenotype correlation. The limited number of microdeletions characterized per study, the imprecise definition of their boundaries, and the lack of appropriate control patients, had made it difficult to establish reliable genotype-phenotype correlations.

To further explore the different *NF1* locus rearrangements, and the putative association with such a contiguous gene syndrome phenotype, a large cohort of 70 unrelated *NF1* microdeleted patients was studied. Their microdeletions were accurately characterized using a high-resolution *NF1* custom array comparative genomic hybridization (CGH) targeting the 17q11.2 region. The genotype-phenotype correlation in 58 of these microdeletion patients was statistically evaluated, taking 389 unrelated patients with intragenic *NF1* truncating mutations as a reference group. All NF1 patients were clinically assessed utilising the same standardized

questionnaire collection protocol. Our results demonstrate that *NF1* microdeletion patients have a significantly higher incidence of learning disabilities and facial dysmorphism than patients with intragenic *NF1* mutation, after adjusting for age and sex.

MATERIAL AND METHODS

Study samples

A initial panel of 21 *NF1* microdeleted patients was identified through a comprehensive mutation screening of 561 NF1 index cases, enrolled between 2002 and 2005 in the French Clinical Research Program entitled "Study of expressivity of neurofibromatosis 1: constitution of a phenotype-genotype database" (coordinator: Pr. P. Wolkenstein, Henri Mondor Hospital, Créteil, France) to constitute the so-called 'NF-France database', as previously described (Sabbagh et al. 2008). The mutation screening of the *NF1* gene was performed using a variety of *NF1* gene screening methodologies that included, DNA and RNA sequencing, for small lesions, polymorphic microsatellite marker analysis and multiplex ligation-dependent probe amplification (MLPA) or real-time PCR-based gene-dosage analysis to permit microdeletion assessment**,** as previously described (Pasmant et al., 2008; Upadhyaya et al., 2008). Among the 561 NF1 index cases, 512 individuals presented a *NF1* alteration, 2 showed a *SPRED1* (sprouty-related, EVH1 domain containing 1; NM_152594) mutation, and no *NF1* and *SPRED1* mutation was identified in the 47 remaining patients, as previously described (Pasmant et al., 2009b). Among the 512 index cases with a fully characterized *NF1* mutation, 21 patients (4%) presented a *NF1* microdeletion, in accordance with previous data (Kluwe et al., 2004). A second cohort of 49 *NF1* microdeleted patients was selected from routine *NF1* genotyping at Beaujon Hospital (Clichy, France) and at Institute of Medical Genetics (Cardiff, UK).

In total, 70 unrelated NF1 patients harbouring a *NF1* microdeletion were included in this present study, of which 50 patients (79%) had a *de novo* microdeletion and 13 patients (21%) had inherited their microdeletion. Relevant inheritance data was unavailable for the remaining 7 patients.

The reference control group for this genotype-phenotype correlation study involved the use of non-deleted NF1 patients with characterized *NF1* intragenic truncating mutations selected from the NF-France database. Only patients with constitutional *NF1* nonsense or frameshift mutations were included as it is assumed all such mutations would lead to the premature truncation of neurofibromin. A total of 389 NF1 patients met this criterion and were included in the reference group of non-deleted patients. Patients with missense mutations, in-frame deletions or insertions were excluded from analyses since the functional consequences of such mutations are difficult to assess without functional studies. The 389 patients *NF1* molecular alterations were characterized in the same laboratory (Beaujon Hospital, Clichy, France).

For the 70 microdeleted and 389 non-deleted NF1 patients, the full clinical available information was recorded utilising a standardized questionnaire. All patients included in this study fulfilled the National Institutes of Health (NIH) diagnostic criteria for NF1 (Ferner et al., 2007) and their written informed consent was obtained.

Phenotypes

Fourteen major clinical features of NF1 were selected for consideration for the genotype-phenotype association study, as previously described (Sabbagh et al., 2008). Four were quantitative traits: number of CAL spots (a continuous variable), number of plexiform neurofibromas (a continuous variable), and number of cutaneous and subcutaneous neurofibromas (each classified into one of four semi quantitative categories based on the number of neurofibromas: 0, 1–9, 10–99 and $\geq$ 100). The other ten clinical features were coded as binary variables: presence/absence of skin-fold freckling, blue-red macules, Lisch nodules, macrocephaly, childhood overgrowth, facial dysmorphism, scoliosis, learning disabilities, optic gliomas, and MPNST development. Patients with missing data for a particular feature were coded as "unknown", and were not then considered in models involving that feature. Most features were identified by physical examination, with Lisch nodules being diagnosed, or excluded, by slit-lamp examination; individuals not given a slit lamp examination were coded as "unknown". The presence or absence of optic gliomas was determined by cranial MRI or CT examination with individuals not given cranial imaging being coded as "unknown". Childhood overgrowth was evaluated only in NF1 patients under the age of 18 and was defined as height and/or head circumference at least two standard deviations above the age- and sex-matched population mean. A facial dysmorphism was diagnosed if two or more of the following signs were observed: coarse face, flat occiput/brachycephaly, facial asymmetry, prominent forehead, frontal bossing, ptosis,

downslanting deep set eyes, eversion of the lateral eyelid, epicanthic folds, high and broad nasal bridge, bulbous nasal tip, large and low set ears, malar hypoplasia, wide and prominent philtrum, micrognathia, small pointed chin and low posterior hairline. For scoliosis, only scoliotic curves of >10° were taken into account in our analysis. The diagnosis of learning disabilities was performed on specific testing of cognitive abilities and/or history of scholar difficulties leading to repeating at least one level. Learning deficits most frequently involved visual spatial, visual motor integration skills and language-based skills. The prevalence of many other clinical abnormalities (epilepsy, hydrocephalus, medullary compression by neurofibroma, cerebrovascular complications, renal artery stenosis, pseudoarthrosis, congenital pseudarthrosis of the tibia, dysplastic vertebrae, sphenoid wing dysplasia, xanthogranuloma) were too low (≤ 5%) for meaningful statistical analysis.

The 70 microdeletion and the 389 control patients' phenotypes, collected through a standardized NF1 questionnaire have been deposited in NCBI's Gene Expression Omnibus (GEO) (Edgar et al., 2002) and are accessible through GEO accession number GSE19730 (at: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE19730).

Characterization of *NF1* microdeletions

The 70 NF1 patients' microdeletions were accurately characterized by using a custom high-resolution oligonucleotide array-CGH targeting the 17q11.2 region. The theoretical (design) and practical aspects (procedure) of this custom array-CGH are described in detail elsewhere (Pasmant et al., 2009a). Briefly, we designed a specific array-CGH for the *NF1* gene and its flanking regions by using the custom Agilent Human Genome 15K Microarray (Agilent Technologies, Santa Clara, CA, USA). The array comprised a total of 14,207 oligonucleotide probes spanning the whole of chromosome 17, including 12 314 probes spanning a ~8 Mb interval surrounding the *NF1* locus (~300 kb). This 8-Mb interval spanned the *NF1* gene region and included the largest *NF1* atypical microdeletions described in the literature (Riva P et al., 2000; Kehrer-Sawatzki et al., 2003; Venturin et al., 2004a; Pasmant et al., 2008). Details of the microarray design, including the 14,207 oligonucleotide probe chromosome locations, has been deposited in NCBI's GEO (Edgar et al., 2002) and are accessible through the GEO accession number GSE19730.

Statistical Analysis

Multiple logistic regression was performed to test the association of each clinical feature individually with the type of constitutional *NF1* mutation (microdeletion vs. intragenic mutation). Each regression model had the covariates of sex and age at examination (as a continuous variable) to control for potential confounding. Odds ratios (OR) and 95% confidence intervals (CI) were also calculated when appropriate. We applied the Bonferroni correction, by which the nominal alpha value is adjusted based upon the number of tests performed, to account for multiple testing. All statistical analyses were performed with StatView 5.0 software (Cary, NC).

RESULTS

Array-CGH characterization of the 70 *NF1* microdeletions

All 70 *NF1* microdeletions were accurately characterized with our *NF1* customised array-CGH platform and consisted of 77% type-1 (54/70), 9% type-2 (6/70), 4% type-3 (new recurrent type) (3/70), and 10% atypical microdeletions (7/70) (Table 1). An estimation of the length of each microdeletion as well as their gene content, was obtained by using the know locations of the last proximal and first distal non-deleted probes (Table 1; Supp. Table S1). A new recurrent ~1.0 Mb microdeletion type, designated here as type-3 microdeletion, was identified in three unrelated NF1 patients (GUE, OLI, and N2603), involving *NF1-REP-b* (but not *NF1-REP-a*) and *NF1-REP-c*. Figure 1 shows the relevant array-CGH profiles for the three recurrent types. The 14,207 array-CGH log2 normalized ratio data of the 70 patients discussed in this publication have been deposited in NCBI's GEO (Edgar et al., 2002) and are accessible through GEO accession number GSE19730 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE19730).

Table 1. Distribution of *NF1* microdeletions types in the 70 studied patients.

	Length	Number of deleted genes (not including NF1)	Inheritance
54 type-1 (77%)	1.4 Mb	14	*de novo*: 85% (40/47) inherited: 15% (7/47)
6 type-2 (9%)	1.2 Mb	13	*de novo*: 67% (4/6) inherited: 33% (2/6)
3 type-3 (4%)	1.0 Mb	8	*de novo*: 100% (3/3)
7 atypical (10%)			
NF00028	830 kb	10	inherited
NF00234	870 kb	6	*de novo*
NF00398	1.0 Mb	9	inherited
NF00358	1.2 Mb	15	inherited
DIE	1.2 Mb	10	inherited
N806	5.5 Mb	~50	*de novo*
DUB	7.6 MB	~100	*de novo*

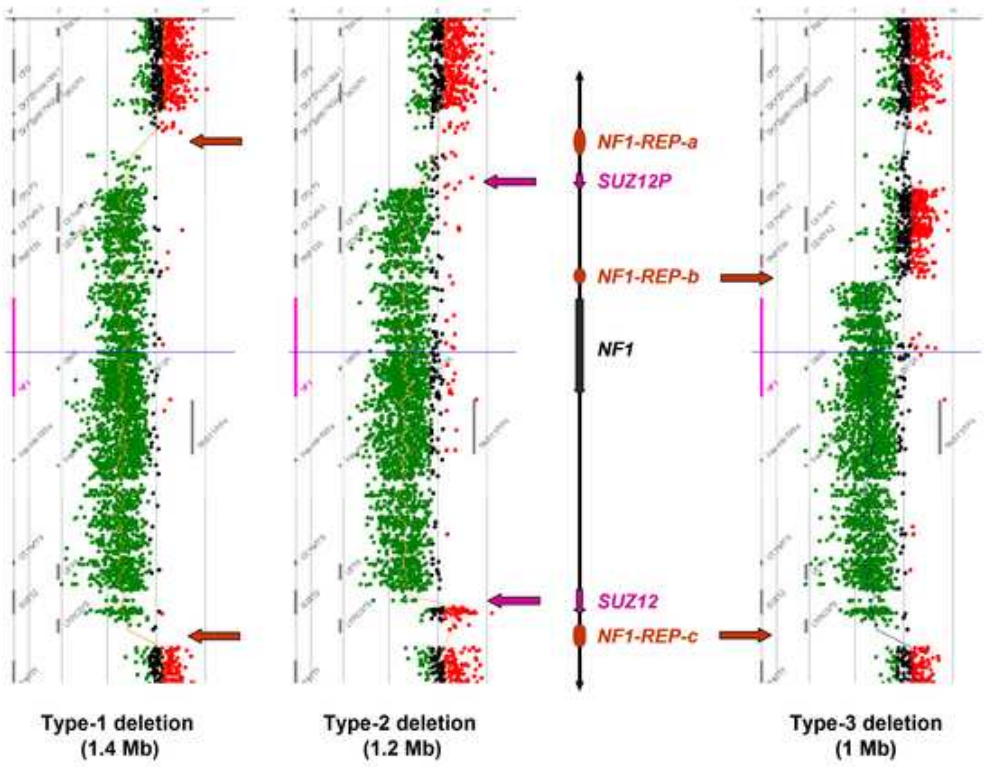

Figure 1. Array-CGH profiles of the three types of recurrent (typical) deletions (including type-1, type-2, and the new type-3) and NF1 locus schematic representation.

Genotype-phenotype correlations

From the 70 microdeleted NF1 patients, 12 individuals were excluded from the statistical analysis of genotype-phenotype correlations: two had the largest atypical microdeletions (DUB: 7.6 Mb and N806: 5.5 Mb), and 10 patients had incomplete phenotype data. In the 58 remaining patients (27 men [46%] and 31 women [53%], mean age at examination = 20.5 ± 17.6 years), there were 44 type-1, 6 type-2, 3 type-3, and 5 atypical microdeletions. The reference group of non-deleted NF1 patients with intragenic truncating mutations (n=389) consisted of 169 men (43%) and 220 women (57%) with a mean age at examination of 33.2 ± 13.9 years.

The clinical characteristics associated with the non-deleted, the microdeleted, and the only type-1 microdeleted NF1 patients, are provided in Table 2.

Table 2. Non-deleted and microdeleted NF1 patients clinical characteristics comparison.

Clinical features	*NF1* non-deleted patients (n=389)[a]	All *NF1* microdeleted patients (n=58)	$P^{b, c}$	*NF1* type-1 microdeleted patients (n=44)	$P^{b, d}$
Cutaneous neurofibromas			NS		NS
0	22.1%	49.0%		51.3%	
1-9	20.1%	17.6%		15.4%	
10-99	34.4%	17.6%		17.9%	
≥100	23.4%	15.7%		15.4%	
Subcutaneous neurofibromas			NS		NS
0	41.8%	54.4%		58.1%	
1-9	40.5%	38.6%		37.2%	
10-99	15.2%	5.3%		2.3%	
≥100	2.6%	1.8%		2.3%	
Plexiform neurofibromas[e]	0.7 ± 1.0	0.6 ± 1.1	NS	0.6 ± 1.1	NS
CAL spots[e]	24.2 ± 16.6	19.3 ± 13.4	NS	20.8 ± 14.5	NS
Skin-fold freckling	92.0%	87.7%	NS	86.4%	NS
Blue-red macules	16.2%	4.0%	NS	2.4%	NS
Lisch nodules	64.2%	43.2%	NS	40.0%	NS
Macrocephaly	13.1%	20.6%	NS	11.5%	NS
Childhood overgrowth[f]	6.3%	30.4%	0.005	22.2%	NS
Facial dysmorphism	10.8%	51.9%	**<0.0001***	54.8%	**<0.0001***
Scoliosis	38.3%	31.5%	NS	31.0%	NS
Learning disabilities	51.9%	80.0%	**0.001***	85.7%	**0.0006***
Optic gliomas	14.9%	16.0%	NS	15.0%	NS
MPNSTs	3.1%	5.6%	NS	7.1%	NS

NS: non significant. [a] Patients with intragenic *NF1* mutations leading to a truncated protein (nonsense or frameshift mutations). [b] *P* values from multiple logistic regression analyses, using sex and age at examination as covariates. Only P values < 0.05 are shown. [c] *P* values resulting from the comparison of non-deleted NF1 patients with all *NF1* microdeleted patients. [d] *P* values resulting from the comparison of *NF1* non-deleted patients with type-1 *NF1* microdeleted patients. [e] Data are expressed as mean ± standard deviation. [f] Only individuals under the age of 18 were evaluated for this trait (63 *NF1* non deleted patients, 23 *NF1* microdeleted patients, 18 *NF1* type-1 microdeleted patients). * Significant after Bonferroni correction for multiple testing.

The prevalence of the different clinical features in non-deleted patients is comparable to previously reported features for the general NF1 population (Huson and Huges, 1994; Friedman and Birch, 1997; North, 2000). When compared to this reference group, the microdeleted patients as a whole (n = 58), had a significantly higher incidence of dysmorphic features (52% vs. 11%, P < 0.0001, OR = 5.3 [2.7-10.4]) and learning disabilities (80% vs. 52%, P = 0.001, OR = 3.3 [1.6-6.7]) at the Bonferroni-corrected significance threshold of 0.004 (0.05 divided by 14 for 14 traits tested individually), after adjusting for age and sex (Table 2). Similar results were obtained when only the type-1 microdeleted patients were evaluated (n = 44), with OR = 5.3 [2.5-11.2] and OR = 5.0 [2.0-12.6] for facial dysmorphism and learning disabilities, respectively. Suggestive evidence for association with

childhood overgrowth was observed when the microdeleted patients were considered as a whole, with this characteristic found to be overrepresented in microdeleted patients compared to non-deleted cases (30% vs. 6%, P = 0.005, OR = 8.3 [1.9-36.4]). This association was however not statistically significant after applying the conservative Bonferroni correction for multiple comparisons. No significant associations were observed for any other clinical features. The apparently higher incidence of cutaneous neurofibromas in non-deleted patients, as compared to microdeleted patients (78% vs. 51%, respectively), is easily explainable by the greater proportion of prepubertal children (< 10 years old) in the latter group (36% vs. 6%). The association of this trait with the presence of an *NF1* microdeletion was indeed not significant (P = 0.57) when the effect of age was properly taken into account in the multiple regression analysis by using age as a covariate.

The proportion of *de novo* cases (which may contain some cases of mosaicism) was significantly higher in microdeleted patients compared to patients with intragenic mutations (79% vs. 43%, Chi Square test, P value < 0.0001). Therefore, somatic mosaicism has to be taken into account in the evaluation of genotype-phenotype correlations as it could explain some of the observed differences between the two groups of patients. However, when the nature (inherited or sporadic) of the *NF1* mutation was introduced in the multiple regression models as a covariate, along with sex and age at examination, the associations of facial dysmorphism and learning disabilities with *NF1* microdeletions (all pooled together) were still significant (P < 0.0001, OR = 4.8 [2.3-10.0] for facial dysmorphism, and P = 0.002, OR = 3.4 [1.5-7.3] for learning disabilities). Significant results were also observed when only the type-1 microdeleted patients were considered (P = 0.0001, OR = 4.9 [2.2-11.1] for facial dysmorphism, and P = 0.001, OR = 5.6 [2.0-15.7] for learning disabilities). Therefore, the association of facial dysmorphism and learning disabilities with *NF1* microdeletions is unlikely to result from the presence of unknown cases of mosaicism in our group of *NF1* patients.

DISCUSSION

The extreme variability of clinical expression in NF1 makes difficult genotype-phenotype correlation, although they are of crucial importance for patients and their families. Hence, solid evidence for the existence of a microdeletion syndrome has awaited a comprehensive phenotype and genotype evaluation of a cohort of *NF1* microdeletion subjects ascertained in an unbiased manner and compared with an appropriate reference population phenotypically evaluated in the same standardized way as microdeleted patients.

The 70 newly characterized *NF1* microdeletion patients described here, the largest cohort ever reported, with the exception of meta-analysis from the published literature (Venturin et al., 2004b), has been both genotyped with an *ad hoc* array-CGH platform, and clinically phenotyped utilising a standardized questionnaire. The microdeletion patients were compared to 389 patients with NF1 diagnosed according to the NIH clinical criteria and presenting intragenic *NF1* truncating mutations. NF1 patients with *NF1* missense mutations, with no *NF1* mutations, and with *SPRED1* mutations were excluded as the NIH criteria cannot reliably distinguish NF1 from NF1-like syndrome caused by *SPRED1* mutations (NFLS; MIM# 611431) (Pasmant et al. 2009b; Spurlock et al., 2009; Messiaen et al., 2009). This selection allowed comparing clinical phenotype consequences of *NF1* locus microdeletions versus the sole *NF1* gene haploinsufficiency (intragenic truncating mutations).

In contrast to previous molecular or cytogenetic techniques, such as multiplex ligation-dependent probe amplification or fluorescence in situ hybridization, our customised array-CGH platform was able to unambiguously differentiate and characterize *NF1* microdeletion types (De Luca et al., 2007; Pasmant et al., 2009a). Notably, the high-resolution of the array-CGH has allowed identification of a new recurrent *NF1* microdeletion type, designated as type-3, with breakpoints in the paralogous regions middle *NF1-REP-b* and distal *NF1-REP–c* (Figure 1). The most common type-1 microdeletion involved two paralogous sequences flanking the *NF1* gene: *NF1* proximal and distal LCR (*NF1-REP-a* and *NF1-REP-c*). A middle LCR (namely *NF1-REP-b*) has been previously identified between *NF1-REP-a* and the *NF1* gene (Jenne et al., 2003; Forbes et al., 2004; De Raedt et al., 2004, 2006) (Supp. Figure S1). These three LCR blocks (*NF1-REP-a*, b, and c) belong to a complex group of paralogs with three components on 17q11.2 (i.e. in the *NF1* microdeletion region: *NF1-REP-a*, *b*, and *c*), six at 17q21 (*NF1-REP-d*, *e*, *f*, *g*, *h*, and *i*), one at 17q24 (*NF1-REP-j*), and another on 19p13 (De Raedt et al., 2004). Interestingly, a genomic microdeletion caused by NAHR between the *NF1-REP-a*, and *NF1-REP-b* repeats and which included *RNF135* and five other genes, but not the *NF1* gene, has been described in an individual with an overgrowth syndrome (Douglas et al., 2007). For the first time, we describe a recurrent type-3 microdeletion in three unrelated NF1 patients (GUE, OLI, and N2603), with breakpoints located in the paralogous sequences *NF1-*

REP-b (located between *RNF135* and *NF1*) and *NF1-REP-c* (Figure 1), consistent with NAHR between these *NF1* medial and distal LCRs. This type-3 microdeletion is ~1 Mb in size and removed the entire *NF1* gene and at least eight other genes: *OMG*, *EVI2B*, *EVI2A*, *RAB11FIP4*, *CR17orf79*, *UTP6*, *SUZ12*, and *LRRC37B*, but not *LRRC37B2*, *CRLF3*, *ATAD5*, *CENTA2*, and *RNF135* (Table 1; Supp. Table S1). It is worth noting that the three type-3 *NF1* microdeletion patients showed facial dysmorphism. Two of them (GUE and OLI) had subcutaneous and plexiform neurofibromas. One of them (GUE) presented learning disabilities while this information was unavailable for N2603 and OLI who were 4 and 3 years old at the examination time, respectively.

The present study confirms in an unbiased manner the existence of a contiguous gene syndrome associated with specific clinical signs in *NF1* microdeletion patients. Two NF1 clinical features, learning disabilities and facial dysmorphism show significant association with *NF1* microdeletions, after adjusting for multiple comparisons. While this finding is consistent with most previous *NF1* microdeletion studies, facial dysmorphism appeared to be much less prevalent in the microdeleted patients herein described than previously reported (52% in this study versus 78% in a meta-analysis of the published literature on the *NF1* microdeletions in 78 patients) (Venturin et al., 2004b). It is noteworthy that the concomitant presence of both learning disabilities and facial dysmorphism was considerably overrepresented in the *NF1* microdeleted patients compared to the non-deleted NF1 cases (45% vs. 7%, $P < 0.0001$, OR = 11.0 [5.6-21.4]). Nevertheless, while patients with large deletions of the *NF1* locus do have a much greater probability to have learning disabilities and facial dysmorphism, it is still impossible to predict the presence of a *NF1* microdeletion based solely on these clinical symtoms, as previously claimed (Tonsgard et al., 1997). No association between these two clinical features was observed in our group of microdeleted patients ($P = 0.45$, after adjusting for age and sex), suggesting that different genetic factors may be involved in the determination of each of these traits. Remarkably, haploinsufficiency of *OMG* gene, mapping in the typical *NF1* microdeletion interval, has been proposed to be involved in the learning disabilities found in *NF1* microdeletion patients (Venturin et al., 2006).

Our study also suggests some evidence of an association of childhood overgrowth with *NF1* microdeletion, although this was not significant at the Bonferroni-corrected threshold. This observation confirms results published by Spiegel et al. who proposed that overgrowth was indeed a component of the phenotypic spectrum of the *NF1* microdeletion syndrome (Spiegel et al., 2005). *RNF135* haploinsufficiency was proposed to be responsible for the overgrowth often observed in individuals with *NF1* microdeletions (Douglas et al., 2007). Interestingly, none of the four microdeleted patients with no *RNF135* deletion (i.e. *RNF135* not included in the microdeletion interval), NF00234, and three type-3 microdeletion patients (GUE, OLI, and N2603), presented childhood overgrowth. It is also of note that no *RNF135* point mutation or deletion has been found in patients with *NF1* intragenic truncating mutation and with overgrowth, ruling out a putative double heterozygosity for mutations in both the *NF1* and *RNF135* genes in these patients (data not shown). Our cohort of *NF1* microdeleted patients had twice as many MPNSTs as did the non-deleted patients (6% vs. 3%), in agreement with previous published results (De Raedt et al., 2003). This finding is however not significant, because of the lack of power from small sample size and the low incidence of this trait. Hence, these two last findings (childhood overgrowth and possibly increased incidence of MPNSTs) warrant be replicated in an independent sample before any conclusions can be drawn.

This study confirms that custom high-resolution array-CGH is a sensitive approach, suitable for accurate characterization of *NF1* microdeletions and able to unambiguously differentiate between the types of microdeletions. Further studies are necessary to identify which gene(s) among the 14 genes deleted in *NF1* microdeletion syndrome, are responsible for learning disabilities and facial dysmorphism. Our study demonstrates however that *NF1* microdeletion patients present a more severe phenotype than that observed in classical NF1 patients, particularly in respect to the presence of learning disabilities. These data must now to be taken into account in NF1 patients' follow-up care.

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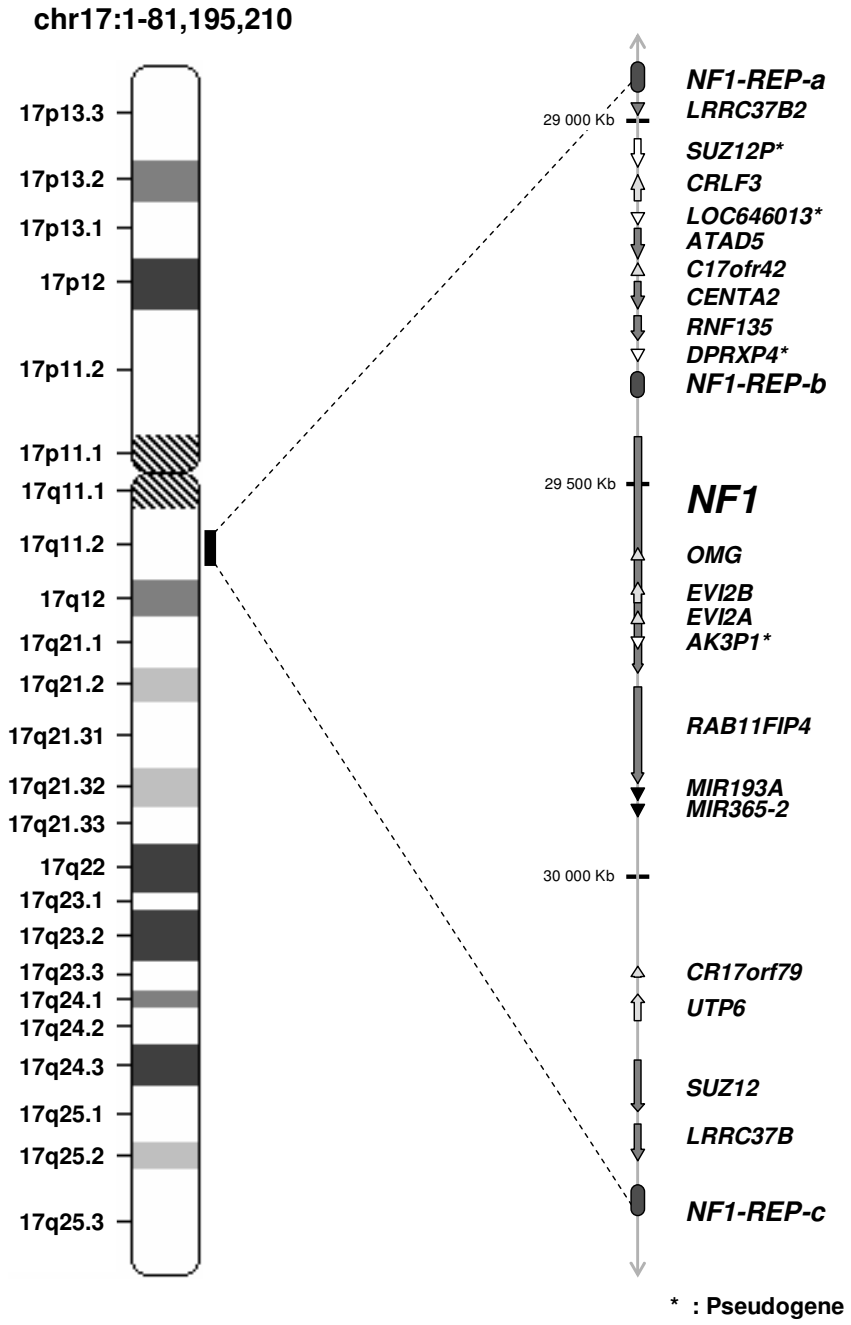

Supp. Figure S1. Schematic representation of the 17q11.2 region. Type-1 1.4 Mb microdeletion encompasses the entire 350-kb NF1 gene, 14 flanking protein-coding genes, four pseudogenes, and two microRNAs.

Supp. Table S1. Gene content of the three typical microdeletions and the < 2 Mb atypical microdeletions.

Microdeletion	Type-1	Type-2	Type-3	Atypical	Atypical	Atypical	Atypical	Atypical	Atypical	Atypical
Patient				NF00028	NF00234	NF00398	NF00358	DIE	N806	DUB
Genes	14	13	8	10	6	9	15	10	~50	~100
SLC6A4										
BLMH										
TMIGD1										
CPD										
GOSR1										
TBC1D29										
LRRC37B2										
CRLF3										
ATAD5										
C17orf42										
CENTA2										
RNF135										
NF1										
OMG										
EVI2B										
EVI2A										
RAB11FIP4										
MIR193A										
MIR365-2										
C17orf79										
UTP6										
SUZ12										
LRRC37B										
RHOT1										