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Research Article

Mutational analysis of the GAP-related domain of the neurofibromatosis type 1 gene in Brazilian *NF1* patients

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Abstract

Neurofibromatosis type 1 (NF1) is a common autosomal dominant disorder caused by mutations in the *NF1* gene. In the present study, a total of 55 unrelated NF1 patients were screened for mutations in the GAP-related domain/GRD (exons 20-27a) by single-strand conformation polymorphism (SSCP). Four different mutations were identified and, taken together, they comprise one nonsense substitution (Q1189X), one deletion (3525-3526delAA), one missense substitution (E1356G) and one mutation in the splice acceptor site (c.4111-1G>A). One novel polymorphism (c.4514+11C>G) and other three putative polymorphisms were also found (c.3315-27G>A, V1146I and V1317A). Genotype-phenotype correlations were investigated, but no particular association was detected.

Key words: gene *NF1*, GRD, neurofibromatosis type 1, mutations, polymorphisms.

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Introduction

Neurofibromatosis type 1 is one of the most common autosomal dominant disorders, affecting approximately 1:3,000 individuals. The main characteristics of the disease comprise multiple neurofibromas, cafe-au-lait skin spots, Lisch nodules and freckling, but, in a minority of patients, other features, such as scoliosis, macrocephaly, short stature, malignancies, and learning disabilities, are also found (Huson and Hughes, 1994).

The *NF1* gene, mapped to 17q11.2 (Barker *et al*., 1987), contains 60 exons and has one of the highest mutation rates described for human genes (Huson and Hughes, 1994). It is transcribed to an mRNA that encodes a protein, neurofibromin. A central region of this protein (exons 20-27a) shows functional and structural homology to the mammalian GTPase-activating protein (GAP). The GAP-related domain (GRD) has been shown to down-regulate $p21^{ras}$ by accelerating the rate of GTP hydrolysis and inhibiting Ras-mediated signal transduction (Martin *et al*., 1990).

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Ten years after the cloning of the *NF1* gene, mutation analysis and diagnostic testing remain a challenge, mainly due to the large size of the gene, the presence of several pseudogenes and the lack of mutational hotspots. Despite these difficulties, more than 400 different *NF1* mutations have been reported (http://www.hgmd.org; http://www.clam.com/nf/nf1gene). Different methodologies have been used to screen mutations in the *NF1* gene. Denaturing high-performance liquid chromatography (DHPLC) was used by Han *et al*. (2001) and De Luca *et al*. (2003), and the mutation detection rates were 97% and 72.5%, respectively. Heteroduplex, FISH, Southern blot, PTT, TGGE and genomic sequencing were also used by Fahsold *et al*. (2000) and Messiaen *et al*. (2000), and up to 95% of mutations were identified. Mutations in the GAP-related domain in lung cancer samples have been screened by Furukawa *et al*. (2003) using SSCP and sequencing, with a mutation and polymorphism detection rate of 8%.

No genotype-phenotype correlation has been detected, except for microdeletions and deletions encompassing the entire gene, or perhaps contiguous genes also, in patients with facial anomalies, great number of neurofibromas and severe developmental delay (Wu *et al*., 1995; Leppig *et al*., 1996; Lopez-Correa *et al*., 2001).

Learning disabilities have also been observed in patients with different inactivating mutations and even in *NF1* mutant flies and mice (Guo *et al*., 2000; Costa *et al*., 2001; Costa and Silva, 2003). Relatively few learning disability data based on IQ tests are reported in molecular studies on neurofibromatosis.

In the present study, we analyzed 55 patients with NF1 for mutations in the GAP-related domain (GRD) of the *NF1* gene.

Materials and Methods

Fifty-five unrelated Brazilian NF1 patients were examined within our multidisciplinary NF1 Program. Thirty-three were familial cases and twenty-two were sporadic. Clinical details were fully documented. Cognitive functions were evaluated using either Wechsler's Intelligence Scale for Children (WISC) or Wechsler's Adult Intelligence Scale (WAIS) tests. All patients gave their informed consent prior to inclusion in the study.

DNA was extracted from peripheral blood leukocytes and amplified by PCR, using the intron-based primers flanking NF1 exons 20-27a (GRD). For exons 20, 22, 23.1, 23.2, 23a, 24 and 26, the primers used for amplification were those described by Li *et al*. (1995) and Van Meyel *et al*. (1994). For exons 21, 25 and 27a, PCR primers were developed using Primer3 software (http://www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). The GenBank accession numbers of the NF1 wild-type sequence were U17683-U17689 (exons 20-27a).

Amplified fragments were then subjected to SSCP analysis. All aberrant SSCP mobility patterns were verified on a second PCR-SSCP analysis, and the DNA samples were automatically sequenced in an ABI 377 PRISM DNA Sequencer (Applied Biosystems). The mutations were also investigated on 100 unrelated alleles, and polymorphisms in 200 control samples.

For molecular modeling we used homology modeling implemented in the program MODELLER (Sali and Blundell, 1993). The atomic coordinates of the GAP-related domain - NF1GRD (NF1-333; residues 1198-1530; PDB access code: 1NF1) were used as template. To generate the complex NF1(E1356G)-Ras, the atomic coordinates of p120GAP (GAP-334; residues 1218-1510; PDB access code: 1WQ1) were used as template (Scheffzek *et al*., 1998).

Results and Discussion

In the 55 unrelated patients screened, four different mutations were identified, of which three are novel: one missense and one nonsense substitutions, and one mutation in the splice acceptor site. Four polymorphisms were also found (Table 1).

The missense mutation (E1356G) of patient 26 (exon 23.2) changed a negatively charged polar for a non-polar amino acid in the peptide. Such a substitution may alter the protein structure and is, thus, likely to be disease-causative. From the information available on neurofibromin sequences of mouse and rat (Genbank L10370 and D45201), this amino acid substitution occurred in a highly conserved position of GRD (identical in human, mouse and rat) and may be essential for GAP function. However, an analysis of the molecular model of the complex NF1(E1356G)-Ras strongly indicated that this mutation has no influence on the interaction between NF1 and Ras, since it is far from the protein-protein interface of the complex (Figure 1).

While the cause of disease in patients with missense mutations is unclear, the significance of stop codons is obvious. In the present study, we identified one nonsense mutation (Q1189X). This mutation (case 28) was identified in exon 21 and is predicted to lead to a severely truncated protein, with only 1,188 amino acids instead of the normal 2,818.

Table 1 - *NF1* mutations and polymorphisms in nine unrelated patients with neurofibromatosis type 1.

Location	Patient	Age (years)	Sporadic/ Familial	Mutation		
				cDNA	Protein	Type
Intron 19 b	34	21	S	c.3315-27G>A		polymorphism
Exon 20	24	27	S	3436G>A	V1146I	polymorphism
Exon 21	28	46	S	3565C>T	Q1189X	nonsense
Exon 21	32	52	S	3525-3526delAA	PTC 1193	deletion
Exon 23.1	8	37	F	3950T>C	V1317A	polymorphism
Exon 23.2	26	12	S	4067A>G	E1356G	missense
Intron 23.2	18	39	F	c.4111-1G>A	?	splice site
Intron 26	16	52	S	c.4514+11C>G		polymorphism
Intron 26	36	37	S	c.4514+11C>G		polymorphism

PTC = premature termination codon.
V = valine, I = isoleucine, Q = glutamine, X = stop codon , A = alanine, E = glutamic acid, G = glycine.

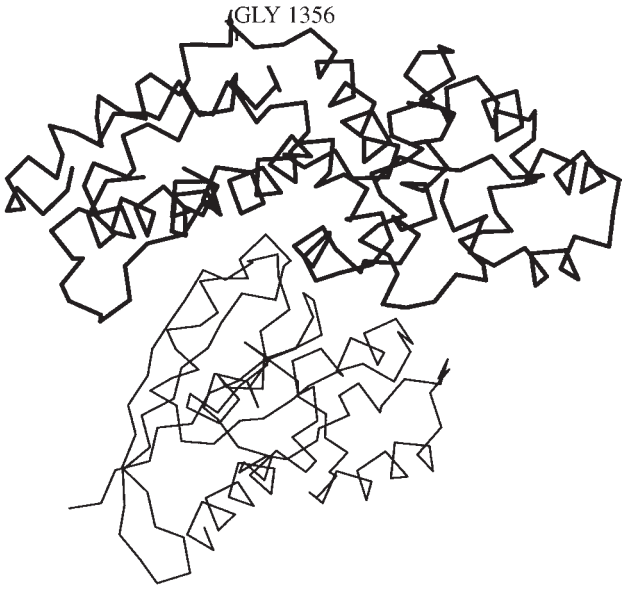

Figure 1 - Cα-backbone for the complex NF1(E1356G)-Ras, showing the G1356 position in the structure. NF1 is drawn with thick lines and Ras with thin lines.

The deletion of two adenines, at nucleotide 3,525 in exon 21 (patient 32), caused a shift in the reading frame, leading to the creation of a premature stop codon at nucleotide 3,580. This mutation may result in the generation of a shortened, non-functional protein of 1,192 amino acids. Recently, the same mutation was reported by Fahsold *et al*. (2000) and Serra *et al*. (2001), but, as in the case of the nonsense mutation, the clinical features of these patients are not available for comparison.

One mutation (case 18) was found in a splice site (c.4111-1G>A) and probably destroyed the acceptor consensus sequence of intron 23.2. Family members of this patient were available for analysis, and the presence of the mutation segregated with the disease. Upadhyaya *et al*. (1997) and Fahsold *et al*. (2000) found different splicing mutations at the donor site of the same intron.

A novel polymorphism (c.4514+11C>G) was detected in intron 26 (patients 16 and 36). This polymorphism was also observed in 3 out of 200 control samples. Other three putative polymorphisms were found in exons 20 (V1146I) and 23.1 (V1317A) and in intron 19b

Table 2 - Clinical features of Brazilian NF1 patients with a predicted prematurely truncated neurofibromin protein (cases 28, 32 and 18) and a missense mutation (case 26) in the *NF1* gene (EEG = electroencephalogram; ECG = electrocardiogram).

	Predicted truncated protein			Missense mutation
	Case 28	Case 32	Case 18	Case 26
Age at diagnosis (years)/Sex	46/F	52/M	39/F	12/M
Cafe-au-lait spots	+	+	+++	+
Freckling	+++	+	+++	++
Neurofibromas	++	+++	++	+
Plexiform neurofibromas	-	-	-	-
Lisch nodules	NA	+	+	+++
Macrocephaly	-	-	-	+
Short stature	+	-	-	-
Mental retardation*	mild	-	mild	borderline
Abnormal EEG	NA	-	+	-
Abnormal ECG	+	+	+	-
Ophthalmological complications	NA	eyelid neurofibroma, retina glioma, pterygium	eyelid and conjuntiva neurofibroma, ptosis, pterygium	-
Skeletal anomalies	cystic lesions	scoliosis, lumbosacral transition vertebra	scoliosis, kyphosis, scalloping	pectus excavatum, osteolytic lesions, cortical fibroma, cystic lesions
Other	-	hemorrhoids, intestinal constipation	headache, hypertension	headache, respiratory problems, dyslexia, spatial orientation problems

M = male, F = female; + = presence of clinical sign, - = absence of clinical sign, NA = not ascertained.
N. of plug signs indicates level of severity:
+ = ≤ 50, ++ = 51-150, +++ = >150 (café-au-lait spots and neurofibromas).
+ = ≤ 4, ++ = 5-8, +++ = >8 (freckling).
+ = ≤ 3 , ++ = 4-6, +++ = >6 (Lisch nodules).
* based on IQ test (IQ = intelligence quotient): (IQ ≥ 85 = normal; 70 ≥ IQ < 85 = borderline; 50 ≥ IQ < 70 = mild mental retardation).

(c.3315-27G>A) in patients 24, 8 and 34, respectively, but in none of the 200 control samples. Both V1146I and V1317A replace a non-polar R group by another non-polar R group and are substitutions often found as polymorphisms in other genes (Miller and Kumar, 2001). The latter polymorphism segregated with the disease in the patient's family and is therefore likely to be located on the same chromosome as the NF1 mutation. Molecular modeling might clarify if V1146I and V1317A affect the structure of the neurofibromin GAP-related domain. However, it is not possible to generate a molecular model for both, because the template NF1GRD (NF1-333; residues 1198-1304 and 1331-1551; PDB access code: 1NF1) has no atomic coordinates in these regions (Scheffzek *et al*., 1998).

This is the first report on mutational screening in a large number of Brazilian NF1 patients. As in previous studies, we were unable to correlate the presence or severity of clinical features and/or mental retardation with the site of the mutation (Table 2). This is not unexpected, given the wide range of clinical variability that has previously been reported, even in different members of the same family. As Messiaen *et al*. (2000) questioned: Do genotype-phenotype correlations in NF1 exist? It is possible that the clinical picture is dependent on many endogenous and exogenous, transitory or lasting, environmental factors. Therefore, in addition to more sensitive methods of mutation detection, studies on gene function are necessary to understand the pathogenesis of this disease. The greatest challenge for the next years will undoubtedly be to link the phenotypic features to the role of neurofibromin and related proteins in growth control and cell differentiation.

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