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ORIGINAL INVESTIGATION

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Mutational and functional analysis of the neurofibromatosis type 1 (*NF1*) gene

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Abstract Neurofibromatosis type 1 (NF1) is one of the most common autosomal dominant disorders. It is caused by mutations in the *NF1* gene which comprises 60 exons and is located on chromosome 17q. The *NF1* gene product, neurofibromin, displays partial homology to GTPase-activating protein (GAP). The GAP-related domain (GRD), encoded by exons 20–27a, is the only region of neurofibromin to which a biological function has been ascribed. A total of 320 unrelated NF1 patients were screened for mutations in the GRD-encoding region of the *NF1* gene. Sixteen different lesions in the NF1 GRD region were identified in a total of 20 patients. Of these lesions, 14 are novel and together comprise three missense, two nonsense and three splice site mutations plus six deletions of between 1 and 4 bp. The effect of one of the missense mutations (R1391S) was studied by in vitro expression of a site-directed mutant and GAP activity assay. The mutant protein, R1391S, was found to be some 300-fold less active than wild-type NF1 GRD. The mutations reported in this study therefore provide further material for the functional analysis of neurofibromin as well as an insight into the mutational spectrum of the NF1 GRD.

Introduction

Von Recklinghausen neurofibromatosis or neurofibromatosis type 1 (NF1) is one of the most common autosomal dominant disorders with an incidence of appoximately 1 in 3000. Associated with the abnormal growth of neural crest-derived cells, it is characterised by the presence of café-au-lait spots, cutaneous or subcutaneous neurofibromas, plexiform neuromas, Lisch nodules and axillary or inguinal freckling (Huson and Hughes 1994). The underlying *NF1* gene, located at 17q11.2, contains 60 exons spanning approximately 350 kb of genomic DNA and encodes a 12-kb transcript (Li et al. 1992; Shen et al. 1996). Since NF1 is characterised both by a higher incidence of certain tumours (Gutmann and Collins 1995) and the loss of heterozygosity in tumour tissue (Legius et al. 1993), it may be concluded that *NF1* possesses the characteristics of a classical tumour suppressor gene.

The gene product, neurofibromin, has a predicted molecular weight of 327 kDa and is widely expressed in many tissues (Daston et al. 1992). A specific region of this 2818-residue cytoplasmic protein (residues 1125–1537, corresponding to exons 20–27a, Gutmann et al. 1993; Gutmann and Collins 1995) is structurally and functionally homologous to the mammalian GTPase-activating protein (GAP) for $p21^{ras}$ and the yeast proteins, IRA1 and IRA2 (Buchberg et al. 1990; Xu et al. 1990). GAP molecules accelerate the hydrolysis of $p21^{ras}$-GTP to $p21^{ras}$-GDP, thereby converting the proto-oncogene from its active to inactive form. The NF1 GAP-related domain (NF1 GRD) has been shown to down-regulate $p21^{ras}$ by accelerating the rate of GTP hydrolysis (Ballester et al,1990; Martin et al. 1990; Xu et al. 1990; Basu et al. 1992; Lowy et al. 1993), whilst studies of NF1 knock-out mice suggest that neurofibromin plays a major role in the down-regulation of *ras* in both neurones and Schwann cells (Kim et al. 1995; Vogel et al. 1995).

The mutation rate in the *NF1* gene is one of the highest reported in any human disorder, with approximately 50% of all NF1 patients having no family history of the disease. To date, more than 80 different inherited *NF1* gene lesions have been identified (Cooper and Krawczak 1996; Shen et al. 1996) including 10 within the NF1 GRD. We report here a further 20 germline mutations within the NF1 GRD of neurofibromin; 14 of these represent novel changes. The missense mutation, R1391S, is associated with a dramatic reduction in GAP activity.

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Materials and methods

DNA manipulations

Genomic DNA prepared from peripheral blood samples was amplified by polymerase chain reaction (PCR) using previously published primer sets (Li et al. 1995). Exon 24 was analysed by heteroduplex analysis (Upadhyaya et al. 1995) whereas exons 20–23, 25 and 26 were subjected to single-strand conformation polymorphism (SSCP) analysis (Upadhyaya et al. 1995). PCR was accomplished in a 10-µl volume containing 5 ng genomic DNA, 20 pmol each primer, 200 µM dNTPs, 50 mM KCl, 10 mM TRIS (pH 8.3), 1.0–1.9 mM $MgCl_2$, 0.01% gelatin and 1 U *Taq* DNA polymerase (Amersham). DNA samples from 70 healthy British Caucasians were used as controls. PCR/direct sequencing was performed as previously described (Upadhyaya et al. 1995).

Site-directed mutagenesis constructs

The R1391S mutation was introduced into the GRD of NF1 by the PCR overlap extension method, as previously described (Poullet et al. 1994). This method makes use of two sets of oligonucleotide primers, a complementary set to introduce the mutation and an outer set for cloning purposes, and two rounds of PCR. The template used for these sets of PCR was pKP22+, a yeast expression vector containing the wild-type GRD (amino acids 1172–1538). The Ser substitution was created using 5′ CATGTTCCTCAGCTTTATCAATC3′ as 5’ sense primer and its complementary sequence as 3′ antisense primer. The outside primers flanked the mutation site, with the 5′ sense primer, GTCACAATGATGGGTGAT, which anneals at residue 1212 within the GRD and encompasses the *Eco*RI site (at amino acid residue 1356) and the 3′ antisense primer, TCAATCACAGCTGCCAGTGTGTATC, which anneals to the last 12 bases of the GRD and a site that was previously cloned into the pKP22+ vector. The first set of PCR products was purified and then used as template for the second round of PCR, using only the outside flanking primers. This 950-bp PCR product was purified and digested with *Eco*RI and *Nde*I, producing a fragment of 260-bp containing the R1391S mutation. This was then subcloned into the same sites of the glutathione *S*-transferase (GST)-fusion expression vector, pGEX-NF1, which contains the same wild-type GRD as pKP22+.

GAP activity of the R1391S construct

Wild-type NF1 GRD and the R1391S mutant were purified as GST fusion proteins as described (Poullet et al. 1994). The GST-NF1-R1391S fusion protein was tested for its GAP activity on H-*ras* in vitro, using the nitrocellulose filter binding method (Basu et al. 1992). This method assays the amount of radioactivity that remains bound to H-*ras* and is proportional to the extent of NF1 GRD-mediated GTP hydrolysis.

Results

A total of 320 unrelated NF1 patients was screened for sequence alterations in the *NF1* gene. Sixteen different lesions in the NF1 GRD region (exons 20–27a) were identified in a total of 20 patients (Table 1); 14 of these were sporadic cases whilst 6 were found in individuals with a family history of the disease. Fourteen of these lesions are novel and together comprise three missense mutations, two nonsense mutations, three mutations in splice sites and six deletions of between 1 and 4 bp.

The exon 20 frameshift mutations detected in sporadic patients N482 and N514 are predicted to generate truncated neurofibromin molecules. Deletions of a T at nucleotide positions 3731 and 3737 in patients N430 and

Table 1 Mutations detected by screening exons 20–26 (encoding the GTPase-activating protein-related domain) of the *NF1* genes of 20 unrelated patients with type I neurofibromatosis. Nucleotide numbering is based on the cDNA sequence of Marchuk *et al.* (1991) (GenBank Accession number M82814)

Exon	Patient number	DNA sequence change	Amino acid substitution/ predicted protein size	Sporadic/ familial	Reference
20	N482	3456delACTC	Premature truncation at residue 1155	S	This report
20	N514	3460delAA	Premature truncation at residue 1192	S	This report
22	N430	3731–3732delT	Premature truncation at residue 1265	F	This report
22	N741	3737delT	Premature truncation at residue 1265	S	This report
22	N817	3826 CGA→TGA	R1276X, premature truncation at residue 1275	S	Heim et al. (1995)
22	N962	3709–2A→G	?	S	This report
23.1	N308	3942 TGG→TGA	W1314X, premature truncation at residue 1313	S	This report
23.2	N250	4084 CGA→TGA	R1362X, premature truncation at residue 1361	F	This report
23.2	N703	4110+1G→A	?	F	This report
23.2	N769	4071–4076delC	Premature truncation at residue 1383	S	This report
23.2	N854	4071–4076delC	Premature truncation at residue 1383	F	This report
23.2	N477	3975–2A→G	?	S	This report
24	N487	4155–4156delA	Premature truncation at residue 1405	S	This report
24	N489	4173 AGA→AGT	R1391S	S	This report
24	N1109	4255 AAG→CAG	K1419Q	F	This report
24	N398	4267 AAG→GAG	K1423E	S	Li et al. (1992)
24	N254	4267 AAG→GAG	K1423E	S	Li et al. (1992)
24	N589	4267 AAG→GAG	K1423E	F	Li et al. (1992)
24	N792	4267 AAG→GAG	K1423E	S	Li et al. (1992)
26	N444	4402 AGT→GGT	S1468G	S	Li et al. (1992)

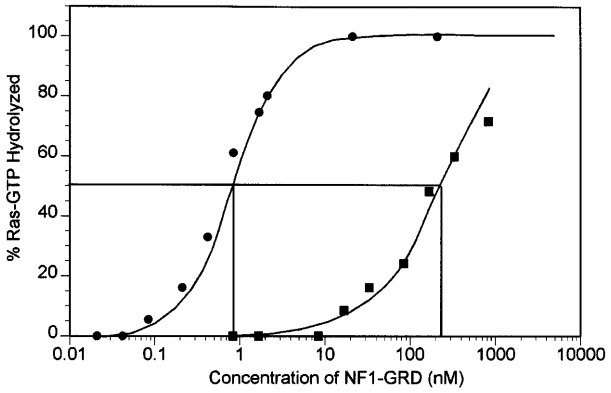

Fig. 1 GTPase-activating protein (GAP) assay of R1391S (■) and wild-type NF1 GAP-related domain (*GRD*) (●) with H-*ras*. R1391S and wild-type NF1 GRD were purified as glutathione *S*-transferase fusion proteins and their GAP activity determined

N741, respectively, are also predicted to result in frameshifts and the production of truncated proteins. The deletion in patient N430 serves to create a new restriction site for *Rsa*I and abolishes a site for *Mae*II; a novel 234-bp *Rsa*I fragment was shown to segregate with the disease in this family (data not shown). Finally, an identical frameshift mutation (4071 del C) was detected in two unrelated patients, N769 and N854.

Three nonsense mutations were also noted in the patients screened: two of these occurred in the hypermutable CpG dinucleotide (Andrews et al. 1996) in codons 1276 and 1362. The CGA→TGA transition at nucleotide 4084 in patient N250 creates a new *Taq*I restriction site which was shown to segregate with the disease phenotype in this three-generation family (data not shown).

The three splice site mutations detected in patients N962, N477 and N703 were located in obligate GT or AG doublets in the exon 22 acceptor, exon 23.2 acceptor and exon 23.2 donor splice sites, respectively. All are likely to result in the generation of aberrant mRNA transcripts.

Of the four missense mutations detected, the 4267A→G transition (K1423E) detected in four unrelated patients from our panel has been reported previously (Li et al. 1992). The other three missense mutations (R1391S, K1419Q and S1468G) are novel and are potentially very valuable for functional studies of the GAP domain. To ascertain the effect of the R1391S mutation on GAP function, the mutant domain was expressed in the form of a glutathione *S*-transferase fusion protein (GST-NF1-R1391S) and tested in vitro for its GAP activity using H-*ras* as a substrate. The R1391S mutant was found to be unable to hydrolyse GTP normally: 50% maximal hydrolysis of H-*ras* GTP was obtained with > 210 nM R1391S NF1 GRD as compared with 0.7 nM wild-type NF1 GRD (Fig. 1). The mutant R1391S NF1 GRD was therefore about 300-fold less active than the wild-type NF1 GRD.

Discussion

The GRD of neurofibromin is the only region so far identified in the protein which has been ascribed a putative function, viz. the down-regulation of $p21^{ras}$ through acceleration of the rate of GTP hydrolysis. The biochemical analysis of missense mutations in the GRD is therefore likely to yield important insights both into GRD structure and function and its interactions with *ras*. To date, ten germline mutations have been reported in the GRD including four missense mutations located at residues 1166, 1276, 1419 and 1423 (Li et al. 1992; Purandare et al. 1994; Heim et al. 1995). This is the first report to describe a systematic mutation screen of the NF1 GRD-encoding exons in a large patient panel and it was successful in yielding a further 14 novel mutations within the domain.

Three of the novel lesions are missense mutations and for one of these, R1391S, we could demonstrate a reduction of over 300-fold in GAP activity. In this context, it is interesting to note that the substitution of arginine with lysine by in vitro mutagenesis results in a significant increase in *ras* interaction (Morcos et al. 1996). That this region is involved in direct interactions with *ras* is evidenced by the fact that synthetic peptides corresponding to neurofibromin residues 1379–1394 are capable of inhibiting NF1 GAP activity (Morcos et al. 1996). The R1391S substitution also results in the loss of interaction between NF1 GRD and *ras* in a two-hybrid assay (M. R. Kim and F. Tamanoi, unpublished). The NF1 patient, N489, with missense mutation R1391S, in addition to café-au-lait spots and neurofibromas, also had scoliosis.

An AAG→GAG transition responsible for the conversion of Lys1423 to Glu was identified in four unrelated patients in this study. This lesion has been reported previously in a familial case of NF1 and as a somatic mutation in three unrelated tumours (Li et al. 1992). This lesion occurs in a region which is highly conserved between human GAP and the yeast IRA proteins. Site-directed mutagenesis/expression studies confirmed that the K1423E mutation resulted in a 200- to 300-fold reduction of GAP activity (Li et al. 1992). Poullet et al. (1994) examined the role of Lys1423 directly by mutating it to all possible alternative amino acids and found that lysine is the only amino acid at this position which is compatible with normal GAP activity. Interestingly, they also found that a second mutation at codon 1434, converting Phe to Ser, could partially restore GAP activity. A further study (Gutmann et al. 1993) tested various GAP-related domain substitutions (including one at codon 1423) and found that while some conferred a reduced ability to regulate *ras* negatively in yeast, the majority either had no effect or were of intermediate effect. Taken together, these studies suggest that a number of amino acids in neurofibromin are critical for the maintenance of GAP activity and that these residues may interact with *ras* in complex ways.

In accord with previous studies of tumour suppressor genes (*BRCA1*, *NF2*, *APC*, among others), the majority of the different mutations reported in this study are predicted

to lead to the premature termination of protein synthesis. Whether or not the four missense substitutions and the three splice site mutations result in the normal synthesis of a stable protein is not known.

In view of the intrafamilial clinical variation observed and the limited number of NF1 patients studied, it is difficult to undertake any reliable assessment of the genotype-phenotype relationship in this disorder. Thus, one of our NF1 patients (N769) developed café-au-lait spots at 3 months, neurofibromas at 9 months and a malignant Schwannoma at age 37 years. He exhibits sensory axonal neuropathy confirmed by sural nerve biopsy (R. Ferner, personal communication). However, patient N854, who possesses an identical 4071delC mutation, manifests classical NF1, characterised by café-au-lait spots, subcutaneous neurofibromas, axillary freckling, a large plexiform neurofibroma, a thoracic spinal deformity and mild cerebral atrophy (M. Splitt, personal communication). In the case of the four unrelated NF1 patients with the same K1423E substitution, no specific or unusual complication of the disease was noted. The absence of any relationship between genotype and phenotype observed so far may be explicable both in terms of the influence of modifier loci (Easton et al. 1993) and by the variable nature, location and developmental timing of the somatic (second hit) mutations which determine the progression and severity of the disease in various tissues.

Sequence analysis of NF1 GRD has revealed a number of highly conserved domains shared by all members of the *ras* GAP protein family. Some invariant residues are conserved between human, *Drosophila* and yeast. These critical invariant residues are apparently essential for GAP function. To assess their functional significance, a limited number of NF1 GRD mutants have been generated and assayed for their ability both to accelerate *ras* GTP hydrolysis and to complement the activity of yeast strains lacking proteins IRA1 and IRA2 (Poullet et al. 1994; Morcos et al. 1996). The mutational data in this study provide further material for functional analysis as well as an insight into the mutational spectrum of the NF1 GRD. It should, however, be noted that neurofibromin possesses an extended region of homology with yeast proteins IRA1 and IRA2, involving exons 16–40 of the *NF1* gene. Sequences outside the strictly defined GRD also exhibit strong evolutionary conservation, which suggests that other regions of neurofibromin may be involved in modulating *ras* GTPase activity and may be responsible for other as yet unknown functions. The functional analysis of mutations in the extended NF1 GRD may well provide further insights into the mechanisms of pathogenesis associated with NF1.

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