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Original Paper

The Guanine Triphosphatase (GTPase) Activating Protein (GAP)-related Domain of the Neurofibromatosis Type 1 Gene is not Mutated in Neural Crest-derived Sporadic Tumours

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We conducted a mutation analysis of the most conserved region of the neurofibromatosis type 1 (*NF1*) gene, the guanine triphosphatase (GTPase) activating protein (GAP)-related domain (NF1 GRD), to which the function of tumour suppressor is attributed. Sixty primary neuroectodermal tumours were analysed. The rationale for the study was based on the likelihood of finding structural alterations resulting in loss of function of this region in tumours of neuroepithelial tissues, where the activity of neurofibromin seems to be crucial in regulating the mechanisms of signal transduction and cell transformation mediated by p21 ras. Following analysis of the whole NF1 GRD sequence, no mutations were identified in the tumours analysed. We conclude that the loss of *NF1* gene tumour suppressor function, that might lead or contribute to the development of malignancies in neuroectodermal tissues, is not due to structural abnormalities of the region of the gene which interacts with p21 ras. © **1998 Elsevier Science Ltd. All rights reserved.**

Key words: neurofibromatosis type 1, neurofibromin tumour suppressor gene, GTPase-activating protein, GAP-related domain neuroectodermal tumour
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INTRODUCTION

NEUROFIBROMATOSIS TYPE 1 (NF1) is one of the most common hereditary diseases predisposing humans to cancer [1]. The *NF1* gene message is ubiquitous; its normal product, neurofibromin, seems to be expressed at the highest level in cells of the nervous system [2]. One of the most important results since the cloning of the *NF1* gene [3, 4], has been the identification of what is still the only known functional *NF1* domain, the NF1 guanine triphosphatase (GTPase) activating protein (GAP)-related domain (GRD), an extremely conserved region of 390 amino acids with structural and functional homology to GTPase activating proteins (GAP) [5]. The GAP proteins downregulate the activity of the *RAS* oncogene by stimulating its intrinsic GTPase activity that converts the active guanine triphosphate (GTP)-bound form of p21 ras to the inactive guanine diphosphate (GDP)-bound form, in the very early stages of the signal transduction cascade. As a GAP protein, neurofibromin is part of the ras-mediated signal transduction mechanisms and can be considered a tumour suppressor gene.

In vitro studies have demonstrated that the *NF1* gene functions through its GRD as a tumour-suppressor gene [6]. This evidence has increased the interest in investigating the possible role of the *NF1* gene in tumorigenesis, both in *NF1* and sporadic cancers. The function of tumour suppressor of the *NF1* gene has been documented by the loss of heterozygosity (LOH) at the *NF1* locus in tumours of NF1 patients [7, 8], and by the compelling evidence of second hit somatic deletions detected in a neurofibrosarcoma and in a neurofibroma of NF1 individuals [9, 10]. Loss of function, documented by the detection of structural aberrations of the *NF1* gene, has been reported in neuroectodermal tumours and in tumour-derived cell lines from NF1 and non-NF1 patients [11–13].

The aim of our study was to confirm the specific role of the NF1 GRD in conferring the function of tumour suppressor to the gene, through the detection of mutations of this region, in primary neural crest-derived tumours. The rationale for the study was based on the likelihood of finding structural

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alterations resulting in loss of function of the NF1 GRD in situations, such as tumours of neuroepithelial tissues, where the activity of neurofibromin, seems to be crucial in regulating the mechanisms of signal transduction and cell proliferation/differentiation mediated by p21 ras.

The study of the NF1 GRD structure so far has mainly focused on the characterisation of exon 24 [11, 14, 15] which, even though the most highly conserved exon, is only a part of the sequence of the *NF1* functional domain. We have conducted a detailed molecular study of the entire sequence of the NF1 GRD.

MATERIALS AND METHODS

Genomic DNA was extracted from 60 primary neuroectodermal tumours, not derived from NF1 patients. The sample included: 14 neuroblastomas, 11 glioblastoma multiforme, 9 astrocytomas, 8 medulloblastomas, 7 ependimomas, 6 peripheral primitive neuroectodermal tumours (PNET), 1 ganglioneuroma, 1 glioma, 1 Ewing sarcoma, 1 meningioma and 1 schwannoma.

Polymerase chain reaction (PCR) amplification of each individual exon of the NF1 GRD was performed with the use of the intronic primers shown in Table 1. The specificity of the products obtained from the amplification conditions, shown in Table 2, and from the use of 30 ng of template DNA per sample, permitted the PCR reactions to be maintained within the exponential phase. These conditions allowed us to discriminate quantitatively between amplifications of 30 and 15 ng of template DNA, and were therefore likely to permit the recognition of a possible hemizigosity for complete loss of one allele. This strategy was chosen as an alternative to the use of a positive control NF1 hemizygous DNA, or normal adjacent tissue control, which were not available.

In order to assess both the quality of the PCR reactions and the presence of altered fragments, due to insertion or deletion mutations, the amplified products were subjected to polyacrylamide gel electrophoresis (PAGE) analysis in 10% acrylamide with 2% cross linker. Single-strand conformation polymorphism (SSCP) analysis, to detect point mutations, was performed on an 8% polyacrylamide gel ($20 \times 20 \times 0.75$ cm), 2% cross linker, with and without 5% glycerol, $0.5\times$ virus-borate/EDTA, 20W constant power, in a water-jacketed electrophoresis chamber. Single- and double-stranded DNA signals were visualised with silver staining.

RESULTS

The amplification products obtained from the tumour DNAs did not show any evidence of hemizygosity due to the loss of one allele on PAGE analysis; no altered fragments

Table 1. *Detail of the polymerase chain reaction (PCR) primers used for the analysis*

Exon	Primer sequence	PCR product (bp)
21	F 5′-ATGTAAGAGAAGCAAAAATT-3′ R 5′-TTGCTATGTGCCAGGGGACTT-3′	329
22	F 5′-TGCTACTCTTTAGCTTCCTAC-3′ R 5′-CCTTAAAAGAAGACAATCAGCC-3′	331
23-1	F 5′-TTTGTATCATTCATTTTGTGTGTA-3′ R 5′-AAAAACACGGTTCTATGTGAAAAG-3′	281
23-2	F 5′-CTTAAGTCTGTATAAGAGTCTC-3′ R 5′-ACTTTAGATTAATAATGGTAATCTC-3′	259
23a	F 5′-AGCCAGAAATAGTATACATGATTGGGT-3′ R 5′-CTATTTTCTGCCAGAATTAGTAGA-3′	447
24	F 5′-CAAACCTTATACTCAATTCTCAACTC-3′ R 5′-AAGGGGAATTTAAGATAGCTAGATTATC-3′	236
25	F 5′-AACCCTGTTTTATTGTGTAG-3′ R 5′-GTAAGTGGCAAGAAAATTAC-3′	140
26	F 5′-TGAAAATTCTAATGACTTTG-3′ R 5′-TCTAAATTTAAACGGAGAGT-3′	238
27a	F 5′-CCAGTTACAAGTTAAACAAATGTG-3′ R 5′-CTAACAAGTGGCCTGGTGGCAAAC-3′	300

Primers for exons 22, 23-1, 23-2, 23a: NNFF International NF1 Genetic Analysis Consortium. Exon 24 [11], exon 27a [16].

Table 2. *Polymerase chain reaction (PCR) amplification of NF*1 *GRD exons: specific conditions*

		Thermal cycle (temp./time)					
Exon	Buffer	Initial denaturation	Denaturation	Annealing	Extention	Final extention	Cycles
21	B	93°C/5 min	93°C/1 min	60°C/1 min	72°C/1 min	72°C/5 min	30
22	B	93°C/5 min	93°C/48 sec	53°C/48 sec	72°C/1 min 30 sec	72°C/5 min	30
23(1)	B	93°C/5 min	93°C/48 sec	53°C/48 sec	72°C/1 min 30 sec	72°C/5 min	30
23(2)	B	93°C/5 min	93°C/48 sec	53°C/48 sec	72°C/1 min 30 sec	72°C/5 min	30
23a	A	93°C/5 min	93°C/48 sec	53°C/48 sec	72°C/1 min 30 sec	72°C/5 min	30
24	B	95°C/7 min	95°C/1 min	58°C/1 min	72°C/1 min	72°C/10 min	35
25	B	93°C/5 min	93°C/48 sec	60°C/48 sec	72°C/1 min 30 sec	72°C/5 min	30
26	A	93°C/5 min	93°C/48 sec	53°C/48 sec	72°C/1 min 30 sec	72°C/5 min	30
27a	B	93°C/5 min	93°C/1 min	60°C/1 min	72°C/1 min	72°C/5 min	30

Buffer A, 6.7 mM $MgCl_2$, 16 mM $(NH4)_2$ SO_4 10 mM 2-mercaptoethanol, 65 mM Tris-HCl pH 8.8, 170 μg/ml bovine serum albumin. Buffer B, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris HCl pH 8.3, 0.1 mg/ml gelatine.

representing deletion/insertion mutations were detected in any of the samples. The SSCP analysis of these amplified fragments did not show alterations of the migration pattern of the single-stranded DNA due to point mutations that could be represented by deletions, insertions or substitutions of a single base.

DISCUSSION

The strategy adopted for the mutation analysis of the functionally most relevant part of the *NF1* gene enabled us to identify structural alterations of medium to small size, including point mutations, and allowed the identification of a state of hemizygosity at the level of the DNA fragment analysed. A search for small size mutations was considered appropriate as the *NF1* gene seems to be more frequently altered by point mutations (NNFF International NF1 Genetic Analysis Consortium). Several techniques have been determined for the efficient detection of point mutations and no 'best method' has yet been found; nevertheless, the SSCP analysis is probably the most widely used technique, having proved to be a relatively simple but effective procedure. Reduction to hemizygosity is one of the crucial steps of the process leading to the loss of function of a tumour suppressor gene, according to the retinoblastoma gene recessive model, which would apply to the *NF1* gene. We approached the problem of a semiquantitative PCR analysis of the NF1 GRD exons, setting experimental conditions that, by keeping the reactions within the limits of the exponential phase, would allow us to detect a change of 2-fold in the starting template DNA and therefore to discriminate between the presence of one or two copies of the gene.

The failure to detect mutations of the NF1 GRD with this analysis, conducted on a large sample of primary neural crest-derived tumours, suggests that structural alterations of the NF1 GRD are not common, if at all involved, in the process of tumorigenesis that may be related to loss of the *NF1* tumour suppressor function, at least in tissues of neuroectodermal origin.

In spite of the fact that the GRD is not frequently mutated in tumours, its GAP activity could indeed account for the well-established function of tumour suppressor of the *NF1* gene. None the less, the mode of action of neurofibromin is still controversial: several studies have demonstrated an increase of the active GTP-bound form of p21 ras in response to a reduction or absence of neurofibromin [17, 18], but no correlation between neurofibromin levels and the proportion of p21 in the active GTP-bound state has been demonstrated in neuroblastomas [19] and in sporadic melanoma tumour cell lines [13]. This evidence seems to suggest that, in some neural crest-derived cell types, neurofibromin may not be a negative regulator of p21 ras, but rather a downstream effector of it in the differentiation pathway. Alternatively, the role of *NF1* as part of the mechanisms of ras-mediated signal transduction, might be independent of its anti-oncogene function.

The fact that we did not find mutations of the NF1 GRD in any of the tumour samples analysed seems to favour this latter hypothesis. We do not know whether in the 60 tumours analysed neurofibromin was actually not functional or could have been downregulated in any way. We can, however, conclude that the process of tumorigenesis did not alter the structure of the NF1 GRD which is expected to interact with ras, regulating its differentiation/proliferation pathway in neural crest-derived tissues.

In conclusion, our data seem to suggest that, if neurofibromin plays a role in tumour suppression in neuroectodermal tissues, this function may not be ras-mediated.

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