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ARTICLE

Exploring the somatic *NF1* mutational spectrum associated with NF1 cutaneous neurofibromas

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Neurofibromatosis type-1 (NF1), caused by heterozygous inactivation of the *NF1* tumour suppressor gene, is associated with the development of benign and malignant peripheral nerve sheath tumours (MPNSTs). Although numerous germline *NF1* mutations have been identified, relatively few somatic *NF1* mutations have been described in neurofibromas. Here we have screened 109 cutaneous neurofibromas, excised from 46 unrelated NF1 patients, for somatic *NF1* mutations. *NF1* mutation screening (involving loss-of-heterozygosity (LOH) analysis, multiplex ligation-dependent probe amplification and DNA sequencing) identified 77 somatic *NF1* point mutations, of which 53 were novel. LOH spanning the *NF1* gene region was evident in 25 neurofibromas, but in contrast to previous data from MPNSTs, it was absent at the *TP53*, *CDKN2A* and *RB1* gene loci. Analysis of DNA/RNA from neurofibroma-derived Schwann cell cultures revealed *NF1* mutations in four tumours whose presence had been overlooked in the tumour DNA. Bioinformatics analysis suggested that four of seven novel somatic *NF1* missense mutations (p.A330T, p.Q519P, p.A776T, p.S1463F) could be of functional/clinical significance. Functional analysis confirmed this prediction for p.S1463F, located within the GTPase-activating protein-related domain, as this mutation resulted in a 150-fold increase in activated GTP-bound Ras. Comparison of the relative frequencies of the different types of somatic *NF1* mutation observed with those of their previously reported germline counterparts revealed significant ($P=0.001$) differences. Although non-identical somatic mutations involving either the same or adjacent nucleotides were identified in three pairs of tumours from the same patients ($P<0.0002$), no association was noted between the type of germline and somatic *NF1* lesion within the same individual.

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INTRODUCTION

Neurofibromatosis type-1 (NF1; MIM number 162200) is an autosomal dominant tumour predisposition syndrome, affecting about 1 in 4000 people worldwide. NF1 is associated with a highly variable clinical phenotype.[1] It results from inactivating mutations in the 17q11.2-located *NF1* gene that leads to the functional loss of its protein product, neurofibromin. The *NF1* gene spans ~282 kb of genomic DNA, contains 61 exons (four of which are alternatively spliced) and encodes a ~9-kb mRNA transcript. Neurofibromin is a highly conserved RAS-GTPase-activating protein (GAP) that is directly involved in the regulation of Ras signalling.[2–4] It downregulates Ras activation in the cell, thereby also downregulating the multiple downstream effectors activated by Ras, including the PI3K and the mitogen-activated kinase signalling cascades, which are involved in regulating cellular proliferation, DNA synthesis and apoptosis.

NF1 is a classic tumour suppressor gene and, consistent with Knudson's 'two-hit hypothesis', all patients harbour both a normal and a dysfunctional *NF1* gene copy, the latter containing the inherited (germline) mutation. Tumours arising in such patients contain a subpopulation of cells manifesting biallelic inactivation of the *NF1* gene as a consequence of an acquired somatic *NF1* mutation. Whereas the patient-associated *NF1* germline mutational spectrum has been fairly well characterised (at least 1290 different *NF1* gene mutations had been identified by August 2011; Human Gene Mutation Database (HGMD)),[5] comparatively few somatic *NF1* mutations have so far been identified in NF1-associated tumours. This paucity is mainly due to the inherent difficulty in detecting such somatic mutations as a consequence of the cellular heterogeneity of the tumour tissue, but is also a consequence of the relatively small number of benign NF1 tumours analysed to date.[6]

One of the most characteristic clinical features manifested by NF1 patients is the growth of benign peripheral nerve sheath tumours (neurofibromas) in the skin. Whereas cutaneous neurofibromas are present in almost all adult NF1 patients,[7] plexiform neurofibromas (PNFs), a more diffuse type of tumour, are present in only 30–50% of patients. However, 10–15% of PNFs are transformed into malignant peripheral nerve sheath tumours (MPNSTs), a major cause of mortality in NF1.[8] Cutaneous neurofibromas usually appear during adolescence, although they may occasionally occur at an earlier age. The marked variability in neurofibroma number frequently observed between affected individuals from the same NF1 family has led to the suggestion that modifying loci might also be involved in tumour development.[9] The neurofibromas themselves exhibit extensive

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cellular heterogeneity, being composed of hyperproliferative Schwann cells, fibroblasts, mast cells and perineural cells, but it is the Schwann cells specifically in which the *NF1* gene becomes biallelically inactivated in cutaneous neurofibromas.[10,11]

Currently, our understanding of the biological mechanisms underlying NF1 tumourigenesis is rather limited, although recent studies in mouse have (i) confirmed a direct role for the tumour microenvironment and (ii) identified skin-derived precursor cells as the cell of origin for cutaneous neurofibromas.[12] In studies of NF1 patients, somatic mutations affecting a number of other tumour suppressor genes, including *TP53*, *CDKN2A* and *RB1* (variously involved in cell cycle regulation, DNA synthesis and apoptosis), have also been identified in MPNSTs,[13–17] PNFs[18,19] and those cutaneous neurofibromas that have been removed from NF1 patients who carry a particularly high tumour burden.[20]

It is currently unclear (i) why some types of neurofibroma are present from birth, whereas others develop only during adolescence, (ii) what initiates neurofibroma growth and (iii) what determines the number of tumours that develop in a given individual. The acquisition of detailed information on the type, frequency and intragenic location of somatic mutations in the *NF1* gene in a sizeable cohort of patients represents a first step towards improving our understanding of the molecular mechanisms underlying NF1 tumourigenesis. To this end, in the most comprehensive analysis of its kind performed to date, we have screened the *NF1* gene both for loss-of-heterozygosity (LOH) and for microlesions in genomic DNA from 109 cutaneous neurofibromas derived from 46 unrelated patients.

MATERIALS AND METHODS

Patient samples

The study samples comprised a total of 109 cutaneous neurofibromas excised from 46 unrelated patients who displayed the requisite NIH clinical diagnostic criteria for NF1.[21] These samples comprised 94 primary tumour samples taken from 43 patients, plus 15 Schwann cell lines cultured from individual neurofibromas excised from the remaining three patients. Primary tumour tissue was carefully macrodissected away from adjacent normal skin. DNA was then extracted from tumour tissue, cultured Schwann cell lines or patient lymphocytes using phenol/chloroform.[11] This study was approved by the local research ethics committee. All patients provided their written informed consent.

Schwann cell culture

The 15 cutaneous neurofibromas used for primary Schwann cell culture were initially cut into two sections; one was used to culture $NF1^{-/-}$ Schwann cells, whereas the other was used directly for somatic mutation analysis. Neurofibroma-derived Schwann cell samples were cultured as previously described.[11,22,23] Immunocytochemistry was performed using a standard protocol.[24]

Analysis of germline and somatic NF1 mutations

DNA from all 94 tumour samples and 15 Schwann cell lines, together with samples of the corresponding patients' lymphocyte DNA, was initially screened for evidence of LOH using a panel of fluorescently tagged polymorphic markers (see below) encompassing the *NF1* gene region (Figure 1). Those samples that were negative for LOH were then screened for (i) microlesions by DNA sequencing (Figure 1; see below) and (ii) intragenic *NF1* deletions/duplications using MLPA (multiplex ligation-dependent probe amplification; see below; RefSeq: NM_000267). Direct sequencing of patient lymphocyte genomic DNA was also used to characterise the underlying germline *NF1* mutations. Total RNA was extracted from Schwann cells.[25] cDNA corresponding to the coding region of the *NF1* gene was made by reverse transcription and PCR amplified in 24 overlapping fragments.[25] Mutations identified at the cDNA level were always confirmed in genomic DNA by direct cycle sequencing. Details of the *NF1* locus

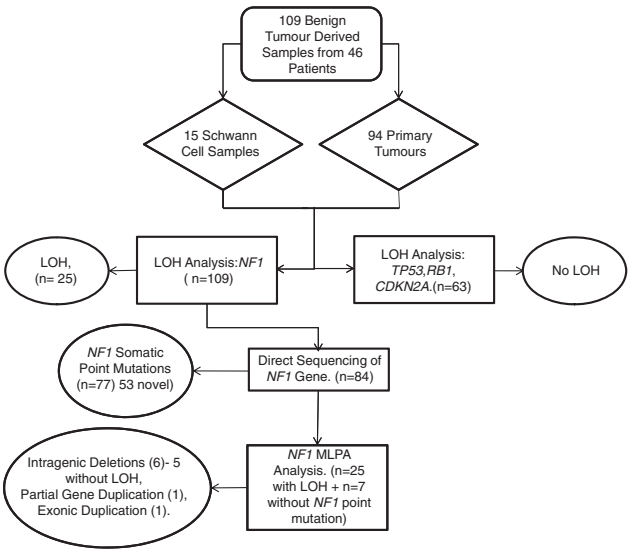

Figure 1 Work flow for detection of somatic mutations involving the *NF1*, *TP53*, *RB1* and *CDKN2A* genes in 109 neurofibromas and neurofibroma-derived Schwann cell lines.

LOH analysis, direct sequencing, *NF1* MLPA analysis, LOH at *TP53*, *RB1* and *CDKN2A* are given under the Supplementary methods.

Functional analysis – site-directed mutagenesis

The oligonucleotide primers for site-directed mutagenesis were designed individually according to the desired mutation using the QuickChange primer design programme (Agilent, Edinburgh, UK). PCR-based site-directed mutagenesis was performed using 10 ng/μl plasmid DNA, 125 ng of each primer, and the following cycle conditions: 95 °C, 30 s; 55 °C, 1 min; 68 °C, 8 min (18× cycles). A volume of 1 μl *Dpn*I restriction enzyme (NEB; 10 U/μl) was directly added to each reaction and incubated at 37 °C for 1 h. Plasmid DNA produced following site-directed mutagenesis was transfected into HEK293 cells using Lipofectamine 2000 (Invitrogen, Glasgow, UK) and following the manufacturers' protocol. Expression of the V5-tagged protein in untransfected controls and mutant cell lines was assessed by western blot.

Ras ELISA

The Ras activation ELISA was performed as described in the manufacturers' protocol (Millipore, Billerica, MA, USA, Cat number: 17-497). Following addition of the chemiluminescent substrate, the Ras ELISA was assessed using a microtitre plate luminometer (Applied Biosystems, Foster City, CA, USA). Relative luminescence (RLU) was evaluated for each sample using WINGLOW software (Perkin Elmer, Waltham, MA, USA) between 5 and 60 min after the substrate was added.

Comparison of germline and somatic mutational spectra

Data sets of somatic mutations (comprising 30 missense/nonsense mutations and 37 microdeletions/microinsertions) identified here and germline mutations (comprising 306 missense/nonsense mutations and 512 microdeletions/microinsertions derived from the HGMD (April 2011 release))[5] in the *NF1* gene were compared in this study. Sequences flanking each *NF1* mutation (either somatic or germline) were screened for the presence of direct and inverted repeats and symmetric elements (of length $\geq$6 bp and $\leq$20 bp apart) by means of complexity analysis,[26] to identify sequences capable of facilitating the formation of non-B DNA structures. Sequences flanking microdeletions and microinsertions were screened for the presence of polynucleotide runs of length $\geq$4 bp known to represent mutational hotspots for duplications and 'de-duplications'[27] via slipped mispairing at DNA replication. In addition, C>T and G>A substitutions within CpG and CpHpG oligonucleotides (where H is either C, A or T) were also recorded for both germline and somatic mutational spectra of

missense and nonsense mutations. The similarities and differences of somatic and germline mutational spectra, with respect to the above features, were assessed by means of Fisher's exact test.

Assessment of functionality or otherwise of identified missense mutations

In an attempt to determine whether (and if so, how) the seven identified somatic *NF1* missense mutations might disrupt neurofibromin structure/function, these amino acid substitutions were analysed using a range of bioinformatic tools. The combined output of these tools was then used to predict which of these mutations is likely to be of functional/pathological significance. Evolutionary conservation of the mutated neurofibromin amino acid residues, across an alignment of 34 placental mammal species, was assessed using *phyloP*.[28] *phyloP* assesses evolutionary conservation/acceleration using a likelihood ratio test (a positive *phyloP* score represents a conserved nucleotide, whereas a negative *phyloP* score indicates that the nucleotide has experienced faster evolution than expected under neutral drift). Protein structure/function disruption and/or the disease-causing potential of missense mutations was evaluated using five different tools: *MutPred*,[29,30] *SIFT*,[31] *PolyPhen2*[32] and Mutation Taster;[33] the potential effect of the missense variants on exon skipping, via loss of exonic splicing enhancers (ESE) and/or gain of exonic splicing silencers (ESS), was ascertained using *Skippy*.[34] *Skippy* outputs a log odds ratio score that quantifies the likelihood that a given nucleotide substitution may lead to exon skipping; a *Skippy* log odds ratio score threshold >2 was used to identify high-confidence variants that may result in exon skipping via ESE loss and/or ESS gain. The ESE and ESS motifs considered in this analysis (Supplementary Table 1) are derived from the NI-ESE and NI-ESS set of exonic regulatory elements.[35] The NI-ESE and NI-ESS sets of exonic regulatory elements motifs have previously been identified as providing the strongest signal for identifying exon-skipping variants.[34] A previously described, neural network was used to assess splice-site disruption and cryptic splice-site activation.[36] The six different lines of evidence (evolutionary conservation (*phyloP*), *MutPred, SIFT, PolyPhen2*, *Mutation Taster* and the splicing predictions) were individually converted to a binary classification (0 or 1), with a '1' representing deleterious (or 'conserved' in the case of *phyloP*) and a '0' representing either a tolerated, non-conserved or a putative passenger mutation. Giving each line of evidence an equal weighting, the majority class ('0' or '1') for each mutation was used to assign the mutation as being either of functional significance (majority of '1's) or not (majority of '0's), or unclassified (equal number of '1's and '0's).

RESULTS

As a first step in this analysis, constitutional *NF1* germline mutations were identified by means of MLPA and DNA sequencing in lymphocyte DNA from 38 of the 46 NF1 patients. This represents an 83% mutation detection rate, which is comparable to previously reported mutation rates for this gene, although it is still significantly lower than the mutation detection rate from the study by Messiaen *et al* (2000).[37–39] These heterozygous inherited lesions comprised 36 truncating mutations and 2 missense mutations, of which 13 were novel (Table 1). All germline mutations were subsequently confirmed by identification in the corresponding tumour DNA samples. Eighteen of the identified *NF1* germline mutations have been previously reported (Table 1, Supplementary Table 2), whereas six have been noted as somatic mutations in neurofibromas, either in this or earlier studies (Supplementary Tables 2 and 3). One germline, *NF1* splice site mutation (c.3113 +1G>A), in this study has also been previously detected as a somatic mutation in a glomus tumour (Supplementary Tables 2 and 3).

A combination of LOH analysis, DNA sequencing and MLPA was used to identify a total of 77 somatic *NF1* point mutations (53 novel) in genomic DNA from the 109 neurofibroma-derived samples (Table 1). These mutations were confirmed by sequencing both DNA strands. None of these lesions were evident in analyses of the corresponding patient lymphocyte DNA. The 77 somatic *NF1* point mutations identified comprised 31 small (1–80 bp) deletions, 22 nonsense mutations, 9 splice site mutations, 8 missense mutations, 6 small (1–2 bp) insertions and an indel (insertion/deletion).

In 25 of the 109 neurofibroma-derived samples, LOH of the *NF1* gene region was evident. In 4 of these 25 cases (in unrelated individuals), a deletion of the entire *NF1* gene was present. The application of MLPA and LOH analysis to 24 of the 25 tumour samples did not reveal any intragenic rearrangement, suggesting that the *NF1*-LOH observed in 24 tumours was probably due to mitotic recombination. In the remaining case (T100), LOH was found to have resulted from the deletion of exons 38–49 of the *NF1* gene (Table 1). Unfortunately, independent confirmation of the results of MPLA analysis by FISH could not be performed, because most of the available tumour samples had been frozen post excision.

In the remaining seven samples, without LOH or an *NF1* point mutation, two single exon deletions (exon 8 and exon 16, respectively), two intragenic duplications (exon 8 and exons 19b-25, respectively) and three large (type 1) genomic deletions (Table 1) were identified.[40]

Analysis of DNA and RNA from neurofibroma-derived Schwann cell cultures allowed us to detect the somatic mutations in four tumours in which the somatic mutation had not been identified by sequence analysis of the original tumour-derived DNA sample. Thus, for example, the somatic *NF1* frameshift mutation (c.1888delG, p.V630fsX), identified in tumour T89.1, was only detectable in DNA from cultured Schwann cells and was not evident in the original tumour DNA sample (Figure 2).

Although seven novel somatic *NF1* missense mutations (A330T, H393D, H393L, Q519P, G629R, A776T, S1463F) were identified here, their pathological relevance was uncertain. None of these substitutions had been noted in any of our previous *NF1* gene mutation screens (involving chromosome 17 from at least 1000 individuals), and all involved amino acid residues that were evolutionarily highly conserved (Table 2). To evaluate the potential functionality of these seven novel missense mutations, we used a suite of bioinformatic tools to assess their impact on protein structure, function and mRNA splicing (Table 2 and Supplementary Table 1). Taking the results together, we surmise that at least four of the seven missense mutations (p.A330T, p.Q519P, p.A776T, p.S1463F) could be of functional/clinical significance (Table 2 and Supplementary Table 1). Two of these missense mutations (p.A330T and pS1463F) were predicted to exert their influence via disruption of protein structure/function (Supplementary Table 1). In addition, the nucleotide substitution underlying p.A776T, located in the first base of exon 15, was predicted to result in the weakening of the splice site (Supplementary Table 1). We then tested our predictions, as far as we could, by performing functional analysis on the p.S1463F missense mutation. This was possible because of its location within the GAP-related domain, as an assay for GAP activity is available. Transfection of cell lines with the p.S1463F mutation resulted in a 150-fold increase in activated GTP-bound Ras (by comparison with cell lines carrying the wild-type NF1 protein) as evidenced by the level of fluorescence recorded by the luminometer (4.5×10^6 RLU; Supplementary Figure 1). Therefore, at least in the case of this GAP-related domain mutation, the functional assay confirmed the bioinformatic predictions.

To explore whether the type of somatic mutation identified in each cutaneous neurofibroma was independent of the type of mutation observed in the germline in the same tumour, the specific combinations of germline and somatic *NF1* mutations identified in the 62 matched lymphocyte/tumour pairs (i.e., where matching germline and somatic mutation data were available) were examined (Supplementary

Table 1 Complete mutation analysis of the *NF1* gene in 109 neurofibromas and neurofibroma-derived Schwann cells from 46 patients

					Somatic NF1 mutations			
Patient ID	*Tumour ID*	*Tissue or Schwann cells*	*Germline NF1 mutation*	*Previously reported*	*LOH*	*MLPA*	*Point mutation*	*Previously reported/ novel*
1	T49.3	Tissue	E1-42: gene deletion	n/a			E24: c.4114_4115delGT	Novel
	T49.1	Tissue					E8: c.1180_1181delTT	Novel
	T49.2	Tissue					E8: c.1177C>G p.H393N	Novel
	T49.8	Tissue					E8: c.1178A>T p.H393 L	Novel
	T49.7	Tissue					E17: c.2953CAA>TAA p.Q985X	Novel
	T49.5	Tissue					E16: c.2446CGA>TGA p.R816X	Maynard *et al*[60]
2	T51.5	Tissue	E1-47: gene deletion	n/a			E11: c.1645_1646delCT p.L549fsX1	Novel
	T51.6	Tissue					E8: c.1179_1180delCT	Novel
	T51.4	Tissue					E16: c.2464GGA>TGA p.G822X	Bausch *et al*[61]
	T51.7	Tissue					E41: c.7285CGA>TGA p.R2429X	Fahsold *et al*[39]
	T51.3	Tissue					E7: c.1062+1G>A	Ars *et al*[62]
3	T63.2	Tissue	Not detected	n/a			E13: c.2088delG	Novel
	T63.8	Tissue					E15: c.2339_2356del18 bp	Novel
	T63.1	Tissue					E15: c.2382 TAT>TAA p.Y794X	Novel
4	T68.1	Tissue	E10b-19b: partial deletion of gene	n/a			E2: c.188insT	Novel
	T68.2	Tissue			LOH: UT172 - I38 206,207; exon 5-3′ region			
	T68.3	Tissue			LOH: UT172 - I38 206,207; exon 5-3′ region			
5	T82.3	Tissue	E12a: c.1754_1757delTAAC p.K583fsX21	Novel			E16: c.2445delG	Novel
	T82.5	Tissue					E35: c.6621_6625 delGTGGA p.Q2207fsX11	Novel
6	T106.1	Tissue	E37: c.6791insA	Fahsold *et al*[39]	LOH: IVS38, 3′NF1-1			
	T106.3	Tissue					E13: c.2033delC	Novel
	T106.4	Tissue					E26: c.4374_4375delCC	Novel
	T106.5	Tissue			LOH: I41 - C3 (3′ UTR)			
	T106.6	Tissue			LOH: J1J2, EVI20, I38, I41, C3C7, 206, 207			
7	T128.1	Tissue	E4b: c.784 CGT>TGT p.R262C	Novel			E8: c.1170delC	Novel
	T128.8	Tissue					E32: c.6055_6056del2 bp	Novel
	T128.17	Tissue					E10c: c.1556CAA>CCA p.Q519P	Novel
	T128.10	Tissue					E4b: c.574CGA>TGA p.R192X	Toliat *et al*[63]
	T128.30	Tissue			LOH: 3′ UTR to 3′ region			
8	T133	Tissue	E16: c.2446 CGA>TGA p.R816X	Maynard *et al*[60]			E31: c.5894insAC p.K1965fsX26	Novel
	T137	Tissue					E31: c.5894insCA p.K1965fsX26	Novel
9	T141.13	Tissue	E13: c.2233delA p.S745fsX2	Novel			E30: c.5729delT p.I1910fsX10	Novel
	T141.4	Tissue					E12b: c.1885GGG>AGG p.G629R	Gasparini *et al*[64]
	T141.5	Tissue			LOH: 202, 12b, IVS27, IVS38, 3′NF1			
10	T143.13	Tissue	E37: c.6792TAC>TAG p.T2264X	Robinson *et al*[65]			E30: c.5730delT p.I1910fsX10	Novel
	T143.2	Tissue					E19a: c.3124insAT delGTAG p.V1042fsX16	Novel
11	T176.1	Tissue	E3-36: gene deletion	n/a			E27b: c.4812 TAC>TAG p.Y1604X	Novel
	T176.2	Tissue					E31: c.5928 TGG>TGA p.W1976X	Novel
	T176.3	Tissue					E23.2: c.4110+1G>C	Fahsold *et al*[39]
12	T191.5	Tissue	E 22: c.3721C>T p.R1241X	Fahsold *et al*[39]			E4b: c.505_525del20 bp	Novel
	T191.9	Tissue					E10b: c.1417delA	Novel
	T191.2	Tissue					E22: c.3721CGA>TGA p.R1241X	Fahsold *et al*[39]
	T191.1	Tissue					E18: c.2991+1 G>A	Purandare *et al*[56]
13	T199	Tissue	E7: c.981_982delGT	Abernathy *et al*[57]	LOH: IVS12, J1J2			
	T199.1	Tissue					E4b: c.528T>A p.D176E	Wimmer *et al*[58]

Table 1 (Continued)

Patient ID	Tumour ID	Tissue or Schwann cells	Germline *NF1* mutation	Previously reported	LOH	Somatic *NF1* mutations: MLPA	Somatic *NF1* mutations: Point mutation	Previously reported/ novel
14	T209.1ii	Tissue	E28: c.4950 C>A p.Y1650X	Novel			E7: c.1062+1G>A	Ars *et al*[62]
	T209.5	Tissue					E25: c4345delA	Novel
	T209.6	Tissue					E37: c.6790_6806del17 bp	Novel
	T209.8	Tissue					E15: c. 2326G>A p. A776T	Novel
	T209.7	Tissue					E10a: c.1318C>T p.R440X	Heim *et al*[59]
	T209.8	Tissue					E14: c.2326-1G>A	Novel
15	T210.2	Tissue	E 42: c.7458delC	Novel		Whole gene deletion		n/a
	T210.4	Tissue				Whole gene deletion		n/a
	T210.8	Tissue				Whole gene deletion		n/a
	T210.4	Tissue				E8: deletion		n/a
	T210.5	Tissue				E8: exon duplication		n/a breakpoints not defined
	T210.1	Tissue					E7: c.1062+1G>A	Ars *et al*[62]
	T210.6	Tissue					E22: c.3870+2T>A	Novel
16	T150.2	Tissue	90 kb gene deletion	Upadhyaya *et al*[60]			E34: c.6409delT	Novel
	T181.1	Tissue					E34: c.6409-6410 delTT	Novel
	T198	Tissue					E42: c.7448delT	Novel
	T181.3	Tissue					E3: c.227insG	Novel
	T34.1	Tissue					E23.2: c 4108C>T p.Q1370X	Novel
	T211.2	Tissue					E7: c.910C>T p.R304X	Wimmer *et al*[58]
	T211.3	Tissue					E17: c 2855T>A p.L952X	Novel
	T181.1	Schwann cells					E27b: c.4706 T>G p.L1569X	Upadhyaya *et al*[60]
	T181.3	Schwann cells					E34: c.6409delT p.L2178fsX	Novel
	T539	Schwann cells				Duplication: Ex19b-25		n/a Breakpoints not defined
	T544	Schwann cells					E23.2: c.3975delG p.R1325fsX	Novel
17	HT1377.1	Tissue	Not detected	n/a	LOH			
	HT1377.2	Tissue			LOH			
18	T22	Tissue	E17: c.2851-2A>G	Osborn and Upadhyaya[61]	LOH: 3′-UTR to 3′-flanking regions			
19	T23.6	Tissue	E41: c.7268delCA p.T2425fsX3	Fahsold *et al*[39]	LOH: EVI20, I38, I41, C3			
20	T89.1	Tissue	E37: c.6789delTTAC p.T2268X	Novel			E12b: c.1888delG p.V630fsX	Novel
21	T98	Tissue	E10b-49: gene deletion	Wimmer *et al*[58]			E20: c.3457_3460del4bp	Upadhyaya *et al*[60]
22	T100	Tissue	E41: c.7267insA p.T2426X	Novel	LOH: I38 to 3′UTR	Deletion: exon 38-49		n/a
23	T109.4	Tissue	Not detected	n/a	LOH: I38, I41, 206 (3′ region)			
24	T139	Tissue	E4a: c.434 delTC p.L145fsX19	Novel			E27a: c.4637 TCA>TGA p.S1546X	Novel
25	T140.4	Tissue	E22: c.3731delT p.V1244fsX22	Upadhyaya *et al*[60]			E41: c.7285 CGA>TGA p.R2429X	Fahsold *et al*[39]
26	T149.5C	Tissue	E34: c.6512insCdelATGAGAGA	Novel			E7: c.988 G>A p.A330T	Novel
27	T157.1A	Tissue	E45: c.7907+3A>T	Novel			E20: c.3491delC p.S1064fsX1	Novel
28	T161.4	Tissue	E10b: c.1466TAT>TGT p.T489C	Spits *et al*[62]			E17: c.2990+1G>A	Wimmer *et al*[58]
29	T164.1E	Tissue	E41: c.7285 C>T p.R2429X	Fahsold *et al*[39]			E23.2: c.4084C>T p.R1362X	Upadhyaya *et al*[60]
30	T170.1A	Tissue	E13: c.2041 C>T p.R681X	Ars *et al*[52]			E12a: c.1797G>A p.W599X	Ars *et al*[62]
31	T183.1	Schwann Cells	E4a: c.373insATGTGTdelG p.R125fsX4	Novel			E42: c.7449 delTGCAGCCACC p.R2498fsX	Novel
	T543.1	Schwann Cells					E:21 c.3568del80 bp p.G1190fsX6	Novel
	T543.2	Schwann Cells			LOH: J1J2-3′NF1			
	T543.3	Schwann Cells					E:26 c.4388 C>T p.S1463F	Novel
32	T186.1C	Tissue	E6-48: gene deletion	n/a			E17: c.2990+1G>A	Wimmer *et al*[58]
33	T214	Tissue	E10b: whole exon del	n/a			E22: c.3826C>T p.R1276X	Heim *et al*[59]
34	T217	Tissue	E2: c.61-1G>C	Novel			E12b: c.1900_1907del8 bp p.I 634fsX1	Novel
35	T167.c	Tissue	Not detected	n/a	LOH: IVS27, IVS38, 3′NF1			
36	T192.1	Tissue	E6-27a: deletion	n/a	LOH: 202,IVS12, J1J2, IVS27			

Table 1 (Continued)

Patient ID	Tumour ID	Tissue or Schwann cells	Germline *NF1* mutation	Previously reported	LOH	Somatic *NF1* mutations: MLPA	Somatic *NF1* mutations: Point mutation	Previously reported/ novel
37	T197	Tissue	E10a: c.1318 C>T p.R440X	Heim *et al*[59]	LOH: J1J2,			
38	T227.2	Tissue	Not detected	n/a	LOH: IVS12, J1J2			
39	T230.2	Tissue	Not detected	n/a	LOH: 202;IVS12, IVS27			
40	T232.2	Tissue	Not detected	n/a	LOH: J1J2			
41	T241	Tissue	Not detected	n/a	LOH: J1J2			
42	T374.5	Tissue	E10a: c.1318 C>T p.R440X	Heim *et al*[59]			E23: c.3916 C>T p.R1306X	Park *et al*[63]
43	HT1335	Tissue	E40: c.7237C>T p.Q2413X	Fahsold *et al*[89]		E16: del		
44	HT1359.2	Tissue	E18: c.3113 +1G>A	Purandare *et al*[56]			E10a: c.1277 G>A p.W425X	Mattocks *et al*[64]
45	T175.1	Schwann cells	E23.2: c.4084 C>T p.R1362X	Upadhyaya *et al*[60]			E12a: c.1733insT p.L578fsX9	Novel
	T175.2	Schwann cells					Ex31: c.5817 C>A p.C1939X	Novel
46	T536A	Schwann c ells	E40: c.7127 132bpdel p.G2376	Novel	LOH: J1J2-IVS38			
	T536B	Schwann cells					E40: c.7169delG p.R2390fsX6	Novel
	T541.1	Schwann cells					E27b: c.4741insG p.G1581fsX19	Novel
	T541.2	Schwann cells			LOH: EV120-3'NF1			
	T541.3	Schwann cells					E12b: c.1888delG p.V630fsX	Novel
	T541.4	Schwann cells			LOH: EV120-3'NF1			

Abbreviations: Del, deletion; fs, frameshift; Ins, insertion; LOH, loss of heterozygosity; MLPA, multiplex ligation-dependent probe amplification; n/a, not applicable (RefSeq: NM_000267); NF1, neurofibromatosis type-1; UTR, untranslated region.
Use of the term 'novel' to describe a mutation implies that it has not previously been reported either in the germline or the soma. Somatic *NF1* mutations were identified in all 109 neurofibroma-derived samples from the 46 patients. In all, 77 of the 109 samples had *NF1* somatic point mutations. LOH was identified in a further 25 patients, in which 24 were likely to be a result of mitotic recombination and 1 of which also had an intragenic deletion. The remaining seven samples had deletions or duplications as identified by MLPA.

Table 4). The type of somatic mutation was found to be independent (P=0.684) of the type of germline mutation (and *vice versa*) in the same tumour when somatic missense, nonsense/frameshift mutations and microdeletions/microinsertions, as well as gross deletions and nonsense/frameshift germline mutations were considered.

Comparison of the relative frequencies of observed somatic *NF1* missense and nonsense mutations, microdeletions, microinsertions and splicing mutations with the frequencies of their *NF1* counterparts in the germline (as reported in HGMD)[5] indicated significant (Fisher's exact test, two-tailed P=0.001) differences. Indeed, inspection of the relative mutation frequencies revealed significantly greater proportions of somatic *NF1* nonsense mutations (29%) and microdeletions (41%), and smaller proportions of *NF1* splicing mutations (12%) and microinsertions (8%) as compared with their germline equivalents (16, 31, 26 and 15%, respectively). The proportion of somatic C>T and G>A missense/nonsense mutations located within CpG and CpHpG was found to be significantly (Fisher's exact test, two-tailed P=0.004) higher (42%) than the corresponding proportion of known *NF1* germline mutations (19%). Somatic microdeletions and microinsertions (31 and 6, respectively) were found to occur in the vicinity of symmetric repeats more frequently than was the case for the germline *NF1* mutations (Fisher's exact test, one-tailed P=0.019). In a similar vein, somatic *NF1* missense/nonsense mutations were found to exhibit a tendency to occur in the vicinity of inverted repeats more frequently than in the germline (P=0.055). Examples of sequence features that could have mediated *NF1* germline and somatic mutations are depicted in Supplementary Figure 2.

No evidence for LOH involving the *TP53, CDKN2A* and *RB1* gene regions was found in any of the 109 tumour and Schwann cell DNA samples.

DISCUSSION

The biological significance of specific somatic *NF1* gene mutations for the tumourigenic process and the possible interplay with their germline counterparts have been difficult issues to address, owing to the paucity of somatic mutational data from neurofibromas and MPNSTs. The main aim of this study was to use a large panel of cutaneous neurofibromas (N=109) from 46 NF1 patients to improve the definition of the somatic *NF1* mutational spectrum.

In all, 53 of the 77 characterised somatic *NF1* mutations identified in this study of cutaneous neurofibromas were novel, having not been previously reported as either germline or somatic lesions (13 of the 38 germline *NF1* mutations detected in the 46 NF1 patients were also novel (see Table 1)).

Schwann cell analysis is known to increase the efficiency of somatic mutation detection,[12] and this was certainly found to be the case in the present study (Figure 2). Unfortunately, the culture of Schwann cells from multiple tumours is a labour-intensive procedure.

The cutaneous neurofibroma-associated somatic *NF1* mutational spectrum characterised in this study appears broadly similar in its distribution of frameshift, nonsense, missense and splice site mutations to those mutational spectra previously reported in the context of PNFs and MPNSTs.[17,18] Indeed, three of the somatic *NF1* mutations characterised here (p.R816X, p.R304X, p.L1569X) have previously been identified as somatic lesions (two of these three mutations are CpG located and therefore compatible with a mechanism of methylation-mediated deamination of 5-methylcytosine) in both benign and malignant tumours.[41–46]

A total of seven novel somatic *NF1* missense mutations were identified during the course of this study. To ascertain their functional/pathological relevance, we used a series of bioinformatic tools

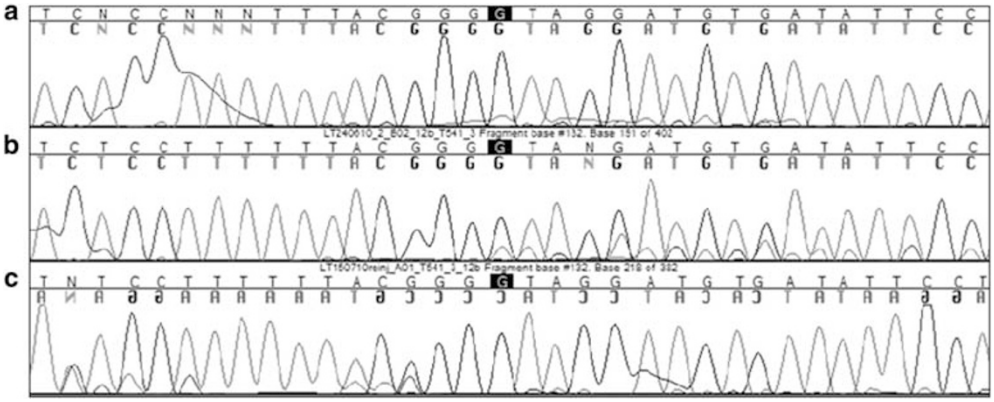

Figure 2 DNA sequence trace of the novel somatic heterozygous frameshift mutation (c.1888delG p.V630fsX) detected in exon 37 of the *NF1* gene from patient T89.1. **(a)** 5′–3′ sequence from neurofibroma tumour tissue, and **(b)** 5′–3′ sequence from Schwann cells cultured from the same neurofibroma. **(c)** 3′–5′ sequence from Schwann cells cultured from the same neurofibroma, illustrating that the G deletion was only detectable in the DNA trace from cultured Schwann cells.

Table 2 Bioinformatic assessment of protein structure/function disruption for the eight identified somatic NF1 missense mutations

Nucleotide substitution	*Amino-acid change*	*Evolutionary conservation* (phyloP)	MutPred *general score (probability of deleterious mutation)*	SIFT *prediction*	PolyPhen2	Mutation taster
c.988G>A	p.A330T	Conserved (2.5)	Deleterious (0.56)	AFFECTS PROTEIN FUNCTION (0.01)	Probably damaging (0.95)	Disease causing (0.91)
c.1177C>G	p.H393D	Conserved (2.5)	Non-deleterious (0.41)	TOLERATED (0.20)	Benign (0.05)	Disease causing (0.78)
c.1178A>T	p.H393L	Conserved (2.0)	Non-deleterious (0.28)	TOLERATED (1.00)	Benign (0.03)	Disease causing (0.88)
c.1556A>C	p.Q519P	Conserved (0.9)	Non-deleterious (0.16)	TOLERATED (2.20)	Possibly damaging (0.24)	Disease causing (0.95)
c.1885G>A	p.G629R	Conserved (2.5)	Deleterious (0.90)	TOLERATED (0.63)	Benign (0.00)	Polymorphism
c.2326G>A	p.A776T	Conserved (2.4)	Deleterious (0.65)	AFFECTS PROTEIN FUNCTION (0.05)	Probably damaging (0.94)	Disease causing (0.99)
c.4388C>T	p.S1463F	Conserved (2.8)	Non-deleterious (0.46)	AFFECTS PROTEIN FUNCTION (0.04)	Possibly damaging (0.59)	Disease causing (0.99)

Abbreviation: NF1, neurofibromatosis type-1.

to assess a number of different parameters including the evolutionary conservation of the affected residues, as well as the potential effect of the mutations on protein structure, function and mRNA splicing. We concluded that at least four of the seven somatic missense mutations (p.A330T, p.Q519P, p.A776T, p.S1463F) could be of functional/clinical significance. To test our predictions, we performed a functional analysis on the only missense mutation located in the GAP-related domain of neurofibromin (p.S1463F). By comparison with cell lines carrying the wild-type NF1 protein, transfection of cell lines with the p.S1463F mutation led to a 150-fold increase in activated GTP-bound Ras. This concurred with our prediction that p.S1463F was likely to be of functional/pathological importance.

The identification of *NF1*-associated LOH (probably associated with mitotic recombination) in 24 tumours is consistent with the results reported in a recent study of neurofibromas that assessed *NF1*-associated LOH in this type of tumour.[47] In our study, MLPA analysis succeeded in identifying only a single intragenic deletion in one neurofibroma, indicating that mitotic recombination was the likely mechanism for LOH in the remaining 24 neurofibromas. Our previous studies found significantly higher levels (approximately 70 and 90%, respectively) of *NF1*-associated LOH in both PNFs and MPNSTs,[17,18] an indication that *NF1*-LOH may be less prominent in benign neurofibromas than in PNFs and malignant tumours. One potential confounding factor here is cellular heterogeneity, but we do not consider that this would have substantially hampered the detection of intragenic deletions and duplications by MLPA in this study. Tumour-associated microsatellite instability was not analysed here, as our previous studies failed to detect significant levels of microsatellite instability in these cutaneous neurofibromas.[44]

Although LOH within the *NF1* gene region was observed in 25 cutaneous neurofibromas, we sought for the remaining intragenic somatic lesions to ascertain whether there might be a relationship between the type of somatic *NF1* mutation found in a given tumour and the type of germline *NF1* mutation in the same patient. Analysis of the somatic *NF1* mutation data (Table 1) indicated that, with the exception of six tumours derived from three unrelated patients (T49.2/T49.3; T133.2/T137; and T150.2/T180.1), all individual cutaneous neurofibromas derived from the same patient were found to harbour independent somatic *NF1* mutations, consistent with the findings of previous studies.[11,20,25] Interestingly, the somatic mutations identified in the patient-paired tumours T49.2/T49.8, T133/T137 and T150.2/T181.1 involve either the same or adjacent nucleotides within the same codons. Thus, for example, neurofibromas T49.2 and T49.8 harbour c.1177C>G and c.1178A>T substitutions, respectively, both involving codon His393. Neurofibromas T133/T137 (c.5894insAC and c.5894insCA) and T150.2/T181.1 (c.6409delT and c.6409delTT) constitute similar examples. The probability of observing mutations in two consecutive nucleotides by chance alone, assuming that the mutation rate (λ) is constant across the *NF1* gene, was estimated by means of a Poisson distribution, with λ defined as the density of observed somatic mutations (both single base-pair substitutions and microdeletions/microinsertions) per nucleotide, and equals $(30+37)/8457=0.008$ (1.26×10^{-4}). We estimate that the probability of observing three such events in an analysis

of the 125 possible pairwise comparisons of pairs of tumours derived from the 17 patients who donated multiple tumours to this study is <0.0002. Several repetitive elements were found in the vicinity of these juxtaposed mutations: for example, an inverted repeat and a symmetric element were found in the vicinity of the c.1177-8 mutations; a direct repeat and run of identical nucleotides span the site of the c.5894 mutations, whereas c.6409 occurs in the vicinity of symmetric elements. These repeat sequences may serve to facilitate the formation of multiple non-B DNA structures,[48] thereby accounting for the hypermutability of these sites (shaded tumour IDs in Table 1). Previous mutation studies have also purportedly found evidence for potential clustering of mutations identified in separate neurofibromas from the same patient,[48,49] but such conclusions have never before received formal statistical support. Although it is possible that the germline *NF1* mutation might influence the location of subsequent somatic *NF1* mutations, our current study on a relatively small number of paired germline somatic mutations have provided no evidence to support this postulate.

Although our previous study[20] found evidence for LOH involving these three loci in cutaneous neurofibromas (albeit infrequently), such LOH was only identified in neurofibromas derived from NF1 patients presenting with very large numbers of such tumours. Only one of the patients in the present study had more than 500 cutaneous neurofibromas, and LOH analysis of his tumours (T210.1–T210.8, Table 1) failed to find any obvious changes at the *TP53*, *CDKN2A* and *RB1* loci.

In summary, this is the first study to provide comprehensive (not merely LOH) *NF1* mutational data from a large cohort of cutaneous neurofibromas. We identified 53 novel *NF1* somatic mutations, which represent a considerable expansion of the known neurofibroma-associated *NF1* somatic mutation spectrum. The results of this study highlight the importance of compiling large data sets of paired somatic and germline mutations, which should eventually help us both to understand the genetic pathways affected in NF1 tumourigenesis and the possible interactions between the germline and somatic *NF1* gene lesions.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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