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ORIGINAL ARTICLE

Analysis of *NF1* somatic mutations in cutaneous neurofibromas from patients with high tumor burden

Laura Thomas · Lan Kluwe · Nadia Chuzhanova · Victor Mautner · Meena Upadhyaya

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Abstract Neurofibromatosis type 1, (NF1) is a complex, autosomal dominant disorder characterized by benign and malignant tumors which result from *NF1* gene mutations. The molecular mechanisms that underlie NF1 tumorigenesis are still poorly understood although inactivation of other modifying loci in conjunction with *NF1* mutations is postulated to be involved. These modifying loci may include deficiencies in mismatch repair genes and elements involved in cell cycle regulation (*TP53*, *RB1*, and *CDKN2A)*. We have analyzed the somatic mutations in 89 cutaneous neurofibromas derived from three unrelated NF1 patients with high tumor burden, by loss of heterozygosity (LOH) analysis of the *NF1*, *TP53*, *RB1*, and *CDKN2A* genes, by assessing microsatellite instability (MSI), by direct sequencing of the *NF1*, *TP53*, and several mismatch repair (MMR*)* genes and by multiplex ligation-dependent probe amplification of the *NF1* and *TP53* genes. The aim was both to assess the possible clonality of these tumors and also to assess the involvement of other potential genetic loci in the development of these neurofibromas. Somatic *NF1* mutations were identified in 57 (64%) of neurofibroma samples. Each mutation was distinct demonstrating the independent origin of each tumor. While somatic LOH of the *TP53* gene was identified in four tumors, no specific deletions or sequence variations were identified. LOH of markers flanking the *RB1* gene was also found in one tumor but no *CDKN2A* mutations were detected. Although evidence of MSI was seen in 21 tumors, no MMR gene alterations were identified. The identification of LOH involving *TP53* and *RB1* loci is a novel finding in benign cutaneous neurofibromas possibly demonstrating an alternative underlying molecular mechanism associated with the development of these benign tumors from this cohort of patients.

Keywords *NF1* · Germline mutations · Somatic mutations · *TP53* · Cutaneous neurofibromas

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L. Thomas · M. Upadhyaya (✉)
Institute of Medical Genetics, School of Medicine,
Cardiff University,
Heath Park,
Cardiff, UK
e-mail: upadhyaya@cardiff.ac.uk

L. Kluwe · V. Mautner
Department of Maxillofacial Surgery,
University Medical Centre Hamburg Eppendorf,
Martinistrasse 52,
20246 Hamburg, Germany

N. Chuzhanova
School of Computing, Engineering and Physical Sciences,
University of Central Lancashire,
Preston, UK

Introduction

Neurofibromatosis type 1, (NF1; MIM 162200), is a complex, inherited, autosomal dominant disorder affecting multiple cell types and body systems in approximately one in 4,000 people worldwide [1]. The *NF1* gene was cloned in 1990 and to date more than 1,200 germline *NF1* mutations have been reported but only ~160 different somatic *NF1* mutations have been reported in cutaneous neurofibromas (Supplementary Table 2). This demonstrates a clear need to expand the *NF1* somatic mutational database to facilitate the identification of common mutations and mechanisms for somatic *NF1* inactivation, to understand

the causes of underlying clinical variability, as well as specific genetic targets for therapies [2].

NF1 is characterized by variable clinical features including: benign cutaneous neurofibromas, pigmentary abnormalities comprising of café au lait macules (CAL), Lisch nodules of the iris and axillary and inguinal freckling, as well as learning difficulties and orthopedic problems [3]. Plexiform neurofibromas of which ~15% become malignant peripheral nerve sheath tumors (MPNSTs), phaeochromocytomas, and optic gliomas are also further complications of this disorder. The *NF1* gene, located at 17q11.2 spans 280 kb of genomic DNA, has 61 exons of which four are alternatively spliced, and which encodes a ~9-kb open reading frame. The large intron associated with exon 27b (61 kb) of the *NF1* gene contains three unrelated genes, *EV12A*, *EV12B*, and *OMG* in which no mutations have been identified in NF1 patients [4]. Neurofibromin, the *NF1* gene product is a large (2,818 amino acid) ubiquitously expressed protein, with the brain and central nervous system exhibiting the highest concentrations. *NF1* is a highly conserved RAS-GAP GTPase tumor suppressor gene involved in regulating Ras signaling [5–7]. Activated Ras has a number of intracellular molecular targets including PI3K, and the mitogen-activated kinase signaling cascade, consequently influencing cell proliferation, DNA synthesis, and apoptosis.

Our understanding of the molecular mechanisms that underlie NF1 tumourigenesis is still relatively limited. In addition to *NF1* mutations, a number of other modifying loci are also thought to be involved [8]. Bi-allelic defects in mismatch repair genes (MMR) can cause instability of microsatellite repeats and are known to be associated with an "NF1-like phenotype". This indicates that *NF1* inactivation may be an important step in malignant progression in mismatch repair deficient cells [9, 10]. In previous studies, microsatellite instability (MSI) has been detected in up to 50% of neurofibromas and at even higher levels in MPNSTs [11, 15]. Animal models which harbor deficiencies in the major MMR genes have also been developed which recapitulate to an extent, NF1 in humans. While zebrafish mutants develop MPNSTs and neurofibromas at low frequencies [16], some MMR knockout animal models do not develop the full spectrum of NF1 features [17, 18]. Therefore, despite some evidence supporting a link between MMR deficiencies and "NF1-like phenotypes", the association remains unclear.

Neoplasms usually arise due to the accumulation of genetic alterations. In predisposition cancer syndromes, a number of genetic abnormalities can occur in pre-malignant lesions which are not present in the normal tissue. Regulatory elements of the cell cycle machinery including *TP53*, *CDKN2A*, and *RB1* are frequently found to be mutated in NF1-associated malignant tumors [19–22]. Abnormal cell cycle arrest mediated by DNA damage and aberrant apoptosis have been found to occur due to loss of *TP53* and *RB1*, and *CDKN2A* loss results in an increase in cell proliferation. Mice carrying cis-linked *Nf1* and *TP53* mutations develop MPNSTs, indicating that accumulation of mutations at various oncogenic loci is important in malignancy. While many studies have demonstrated *TP53* abnormalities in MPNSTs, only two previous studies [23, 24] have identified *TP53* loss of heterozygosity (LOH) in plexiform neurofibromas and there is no indication of the involvement of these genes in the development of benign cutaneous neurofibromas [8, 25–27].

Cutaneous neurofibromas are considered to be the hallmark feature of NF1 and comprise of fibroblasts, mast cells, perineural cells, axons, and Schwann cells [28]. The cell of origin of neurofibromas has been a major subject of debate [29] and it has been unclear how the initial stages of tumourigenesis occur in normal cells and what encourages complete tumor formation following the original genetic lesion. It has been previously determined that only the Schwann cells harbor somatic *NF1* mutations [8] and it was recently established that cutaneous neurofibromas arise from skin-derived precursors (SKPs) with significant contributions from the tumor microenvironment [28]. These models need to be refined, however, and the exact role of the tumor microenvironment and the function of the multitude of cell types found within neurofibromas are still uncertain. Knowledge of genes likely to be altered during neurofibroma formation and identification of cell types which are susceptible to these mutations is therefore crucial to complete comprehension of neurofibromagenesis.

The neurofibromas selected for this present study were tumors derived from NF1 patients with very high tumor burden in which some neurofibromas studied were also present adjacent to each other. Our two basic aims were (1) to determine whether these neurofibromas are clonally derived and result from a single somatic *NF1* mutation, or whether the somatic mutations present in each tumor were distinct independent events and (2) to assess whether somatic mutations in other 'modifying' genes, including the *TP53*, *CDKN2A*, *RB1*, and MMR genes, are also involved in the growth of these neurofibromas from three unrelated NF1 patients with high burden of cutaneous neurofibromas.

Materials and methods

Patients

Three unrelated NF1 patients, two females, aged 44 and 46 (patients 1 and 2, respectively) and one male aged 60 (patient 3) all with a high burden of cutaneous neuro-

fibromas (>550) were recruited for this study. DNA was obtained from 40 cutaneous neurofibromas in patient 1. DNA and RNA were extracted in our laboratory from paired blood and whole tumor tissue from patients 2 and 3 (40 tumors from patient 2 and nine from patient 3). While the exact anatomical location from which the majority of these tumors were excised is unknown, 20 of these neurofibromas were found to be in close proximity to each other, i.e., adjacent tumors which were directly touching and sharing the underlying skin. These 20 tumors are designated with hatched shading in Supplementary Table 1. The remaining tumors are believed to be randomly distributed across the patients' bodies. Samples from all three patients were carefully dissected from the surrounding tumors and skin, and DNA was extracted from tumor tissue using phenol/chloroform [30]. Upon clinical examination, all three patients exhibited the NIH diagnostic criteria for NF1 as well as additional complications as summarized in Table 1. This study gained approval from appropriate institutional review boards and all patients involved provided informed consent.

Analysis of germline and somatic *NF1* mutations

Loss of heterozygosity analysis, MSI analysis, multiplex ligation-dependent probe amplification (MLPA), and direct sequencing of the *NF1* gene was carried out in DNA from each of the 89 neurofibromas to assess the clonal nature of each cohort of cutaneous neurofibromas from the three NF1 patients.

LOH analysis LOH analysis was completed on all 89 neurofibromas and corresponding lymphocyte DNA samples by utilizing a panel of fluorescently tagged microsatellite markers and polymorphic RFLP's spanning the length of the *NF1* gene. Fourteen markers spanning between 17p13 and 17q25, including ten microsatellite markers and four polymorphic RFLP's, were analyzed using an ABI 3100 sequencer and Genotyper and Genescan software (Applied Biosystems, Warrington, UK) [30]. Allelic loss was scored if the area under one allelic peak in the tumor was reduced relative to the other allele, after correcting for the relative peak areas with corresponding

Table 1 Analysis of *NF1* germline and somatic mutations in 89 cutaneous neurofibromas

Patient	Germline mutation	Somatic mutation		Somatic mutation detection rate	MSI	Clinical details
1	E17: c.2875 C>T p.Q959X	7/40	19/40	65%	4/40	>6 CAL spots
						>550 cutaneous neurofibromas
						>2 Lisch nodules
						First degree relative with *NF1*
						Abnormal learning and development
						MPNST in pelvis
2	E10b: c.1413-1414delAG p.KfsX4	15/40	9/40	60%	12/40	>6 CAL spots
						>550 cutaneous neurofibromas
						>2 Lisch nodules
						Sporadic
						Abnormal learning and development
						MPNST in pelvis
3	E36: c.6756+2 T>G	0/9	7/9	78%	5/9	>6 CAL spots
						>550 cutaneous neurofibromas
						>2 Lisch nodules
						Sporadic
						Abnormal learning and development
						Facial plexiform neurofibroma
						Spinal neurofibromas
						Chronic lymphocytic leukemia (B-CLL)
Total		22/89 (25%)	35/89 (40%)	57/89 (64%)	21/89 (24%)	

Full details of somatic mutations identified can be found in supplementary Table 1

lymphocyte DNA. LOH was scored for the polymorphic RFLP's if there was a significant difference in intensity between the bands representing the two heterozygous *NF1* alleles on a 1.5% gel after digestion of the PCR product with the appropriate restriction enzymes. At least two adjacent markers were required to show a reduced signal for LOH to be confirmed.

MSI analysis MSI analysis was completed on all tumor and paired lymphocyte DNA samples with a panel of 10 fluorescently tagged microsatellite markers, an ABI 3100 and Genescan and Genotyper software [31]. A standard marker set was used which included: *D13S153* (13q14), *D5S406* (5p15) *D5S107* (5q11–q13), *BAT26* (2p16) *ACTC* (5q11–q14), *D2S123* (2p16), *D17S250* (17q12), *BAT-25* (4q12), *BAT-40.4* (1p13), and *D5S346* (5q22). The presence of new alleles in the tumor samples which are not found in corresponding blood DNA samples, indicate the presence of MSI.

NF1 MLPA analysis An MLPA assay kit (MRC Holland) containing probes for the entire *NF1* gene, surrounding regions, and control probes was employed to screen for deletions or insertions located throughout the *NF1* gene. An ABI 3100 and excel spreadsheet (NGRL Manchester) were used to characterize the results [32].

Direct sequencing Direct sequencing of all *NF1* exons in genomic DNA and RNA was utilized to detect small sequence alterations in the remaining samples in which neither LOH nor deletions were found. All sequence alterations were fully characterized using an ABI 3730 analyzer (Applied Biosystems) and Sequencher software (Genecodes, USA).

Analysis of somatic MMR, *TP53*, *RB1*, and *CDKN2A* mutations

In view of the exceptionally high burden of these neurofibromas in all three patients and the close proximity of some of the tumors studied, analysis of somatic mutations in additional modifying loci including *TP53*, *RB1*, *CDKN2A*, and MMR genes was completed using LOH analysis and direct sequencing. Genetic aberrations in genes involved in cell cycle regulation, apoptosis, and repair would affect multiple pathways and could contribute to an alternative mutational mechanism underlying the development of these multiple tumors. LOH analysis and direct sequencing of these genes was completed using the same methods and scoring systems that were applied to the analysis of the *NF1* gene.

TP53 All 89 tumor DNA samples and corresponding blood samples were screened by direct sequencing to detect small sequence alterations in exons 4–9 of the *TP53* gene (17p13.1). LOH analysis was performed on four microsatellite markers flanking the *TP53* gene: *D17S796* and *D17S938* (17p13.2), *D17S804* and *D17S520* (17p12) [33], and markers for three polymorphisms intragenic to the *TP53* gene (*Alu1* [34], codon 72 [35], and exon 6 [36]).

RB1 DNA from all 89 tumor samples and matched lymphocyte DNA samples were also analysed for LOH of the *RB1* gene using five microsatellite markers which flank the *RB1* gene: *D13S1557*, *D13S118* (1.2 cM from the 5' region of the *RB1* gene), *D13S153* and *D13S917*, and *D13S119* (5.9 cM from the 3' end of the gene) [37]. Five polymorphic markers within the gene were also used: *RB1.2*, *RB1.3*, *RB1.17*, *RB1.20*, and *RB1.26* [37, 38].

CDKN2A DNA from all 89 tumor samples and matched lymphocyte DNA samples were also screened for LOH using four markers at 9p including: *D9S304* [39], *D9S1748, D9S1751* [40], and *D9S942* [41].

MMR genes Twenty-one tumor DNA samples were sequenced to detect alterations in all exons of the four main MMR genes: *MLH1* (3p21.3), *MSH2* (2p22-p21), *MSH6* (2p16), and *PMS2* (7p22.2).

Bioinformatic analysis

To infer potential mutational mechanisms underlying somatic mutations in the *NF1* gene, sequences flanking these mutations (±20 bp) were screened for the presence of direct and inverted repeats and symmetric elements by means of complexity analysis [42]. Hypothetical mechanisms microdeletion, microinsertion, and indel mutations mediated by these types of repeats have been described previously [43, 44]. In addition, flanking regions were screened for the presence of 37 DNA sequence motifs of length ≥5 bp (plus their complements) known to be associated with site-specific cleavage/recombination, high-frequency mutation, and gene rearrangement [45] as well as various 'super-hotspot motifs' found in the vicinity of micro-deletions/micro-insertions and indels [44].

Results

Analysis of germline and somatic *NF1* mutations

NF1 germline mutations were detected in all the three patients and included: nonsense, 2 bp deletion, and splice

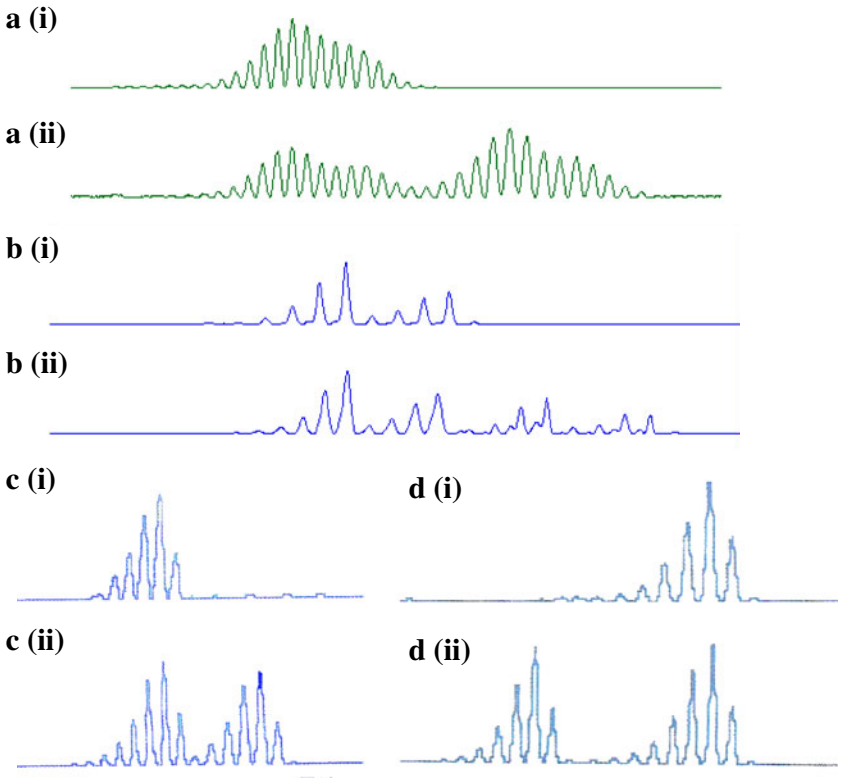

Fig. 1 **a**–**d** Examples of MSI profiles from four different tumors with markers **a** (*i*) *MBAT 40.4* (1p13) in blood and **a** (*ii*) in tumor sample. **b** (*i*) *D17S250* (17q12) in the blood and **b** (*ii*) in the tumor. **c** (*i*) *D5S107* (5q11–q13) in the matched lymphocyte DNA sample and **c** (*ii*) in the tumor sample. **d** (*i*) MSI at marker *D5S406* (5p15) in the blood DNA sample and **d** (*ii*) matched tumor sample

site alteration, the latter two both representing novel changes (Supplementary Table 1). LOH was detected in 22/89 (25%) samples, comprising 18% of tumors in patient 1, 38% in patient 2, and no LOH was detected in patient 3. The extent of LOH ranged from the 5' end of the *NF1* gene to the entire long arm of chromosome 17 (Supplementary Table 1). MSI was observed in 21/89 (24%) tumor samples studied. MSI in these samples was compared to the corresponding lymphocyte DNA samples in which the additional alleles were not present, indicating no MSI was present in the blood DNA (Fig. 1). No deletions were identified in the 89 samples using the *NF1* MLPA kit indicating that LOH in these samples results from mitotic recombination.

Somatic *NF1* mutations were detected in 57 samples screened (22 LOH, 35 small lesions) (Supplementary

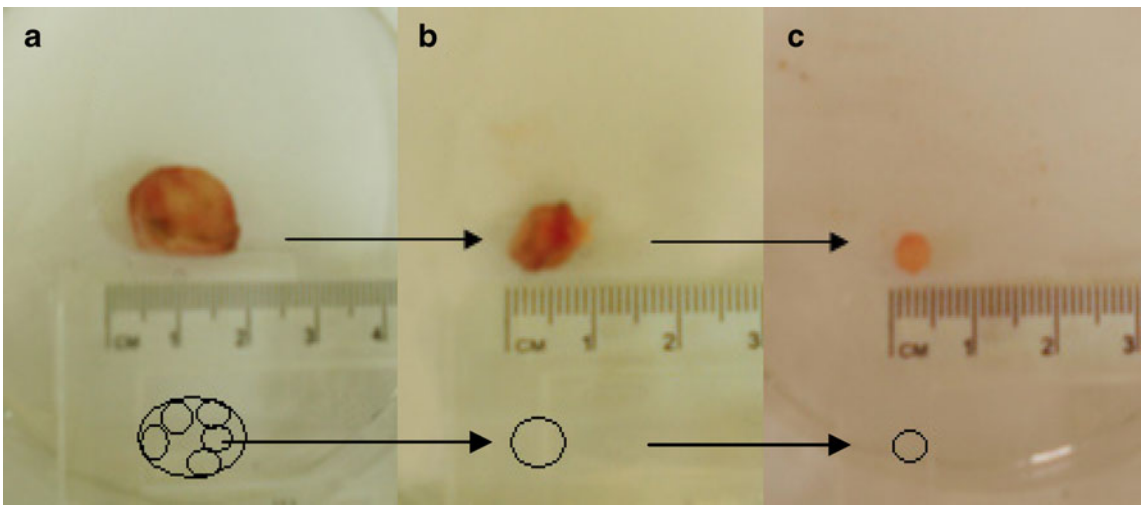

Fig. 2 **a**–**c** Photos and diagrams of the excision of five neurofibromas from one large neurofibroma capsule. **a** Original single neurofibroma capsule (including skin) from which five separate neurofibromas were excised. **b** Section of the neurofibroma pictured in **a** containing one of the five neurofibromas found in this one tumor capsule. **c** Figure showing the same neurofibroma which is depicted in **b** once it has been excised from the surrounding skin and neurofibroma capsule

Table 1). All somatic alterations were absent from the patients germline DNA. The overall mutation detection rate was 64%. Twenty-eight of these mutations are novel. Interestingly, it was found that one individual tumor capsule which was not located adjacent to any other tumor, contained five separate neurofibromas, only two of which demonstrated LOH (Figs. 2 and 3). The somatic *NF1* mutations in each tumor from each individual were independent and the somatic mutational spectrum (Table 1, Fig. 4 and Supplementary Table 1) differs between the three patients as no nonsense and splice mutations were detected in patient 2 and no LOH, nonsense or missense mutations were identified in patient 3.

Analysis of somatic *TP53*, *RB1*, *CDKN2A*, and MMR mutations

No pathogenic *TP53* somatic mutations were identified by direct sequencing. *TP53* LOH was, however, detected in four samples (T440, T473.20, T506.1, and T506.9) at polymorphic markers within the *TP53* gene (*Alu1*, codon 72, and exon 6 polymorphic markers; Fig. 5). One of the four samples showed LOH at all three of these markers. The remaining three samples only exhibited LOH at two of the markers as the codon 72 polymorphism was uninformative (Supplementary Table 3). Additionally, these four samples were also screened for deletions using an MLPA assay kit but no deletions were detected. The level of *TP53* LOH observed at the second allele of each sample after correcting for the relative peak areas with corresponding lymphocyte DNA was between 37% and 66% (*Alu1*), 11% (codon 72; data not shown) and between 35% and 57% (exon 6; Fig. 5). These neurofibromas were not located adjacent to any other tumor and all four tumors had additional *NF1* somatic mutations; comprising of LOH (intron 27–38) and three deletions of 1–114 bp (Supplementary Table 1).

One other sample (T468) also showed LOH of approximately 1 Mb in length at two markers flanking the *RB1* locus (*D13S118* and *D13S917*; Fig. 6, Supplementary Table 4). The mechanism of LOH could not be determined in this case by MLPA as the *RB1* MLPA kit was unavailable in our laboratory. Additionally, this tumor had

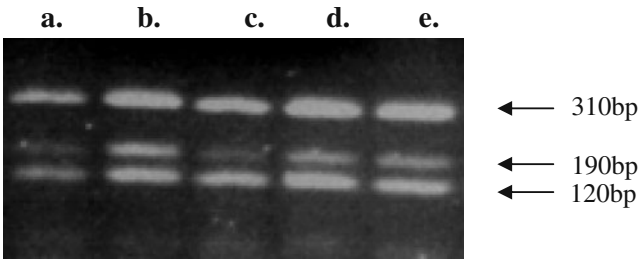

Fig. 3 **a**–**e** LOH of five separate neurofibromas from within the same capsule identified with marker *HHH202*

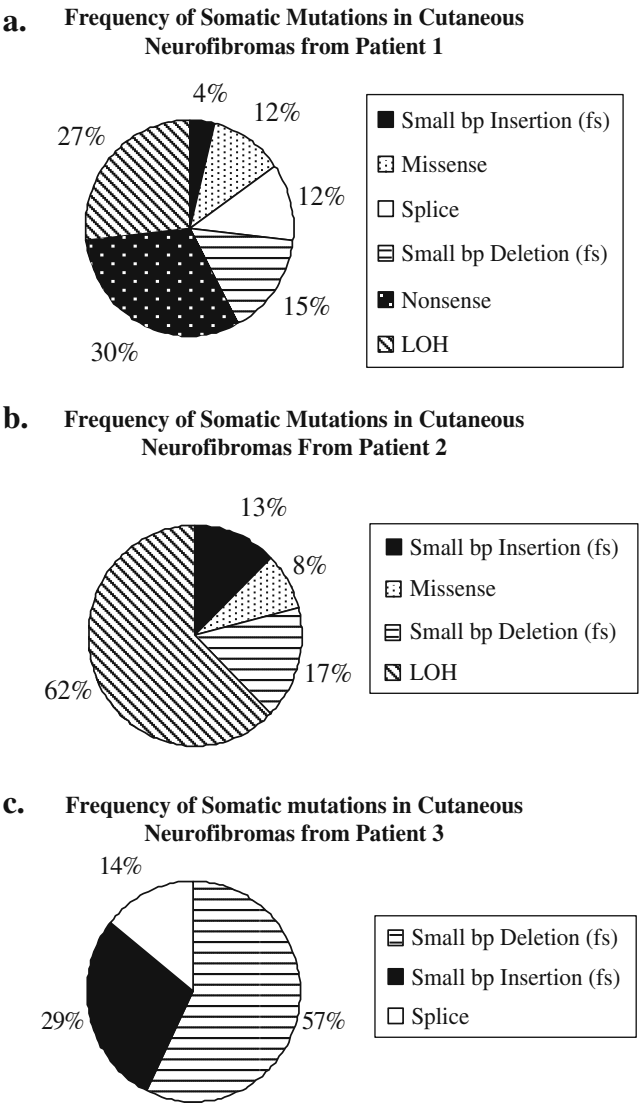

Fig. 4 Somatic mutational spectrum for patients 1–3. **a** *S*omatic mutational spectrum for 40 tumors from patient 1 (Germline mutation, E17: c2875 C>T p.Q959P). **b** *S*omatic mutational spectrum for 40 tumors from patient 2 with an absence of nonsense and splice site mutations (Germline mutation, E10b: c.1413-1414delAG p.Kfsx4). **c** *S*omatic mutational spectrum for nine tumors from patient 3 with an absence of LOH, nonsense, and missense mutations (Germline mutation, E36: 6756+2 T>G)

an *NF1* nonsense mutation and was also not located adjacent to any other tumor (Supplementary Table 1).

No *CDKN2A* LOH was identified at any of the markers that were analyzed. Twenty-one samples were found to have MSI and their DNA was sequenced for the four main MMR genes but no pathogenic somatic mutations or polymorphic changes were identified.

Bioinformatic analysis

Fourteen out of 19 mutations (74%) found in the patient 1 were single base pair substitutions, apparently mediated by

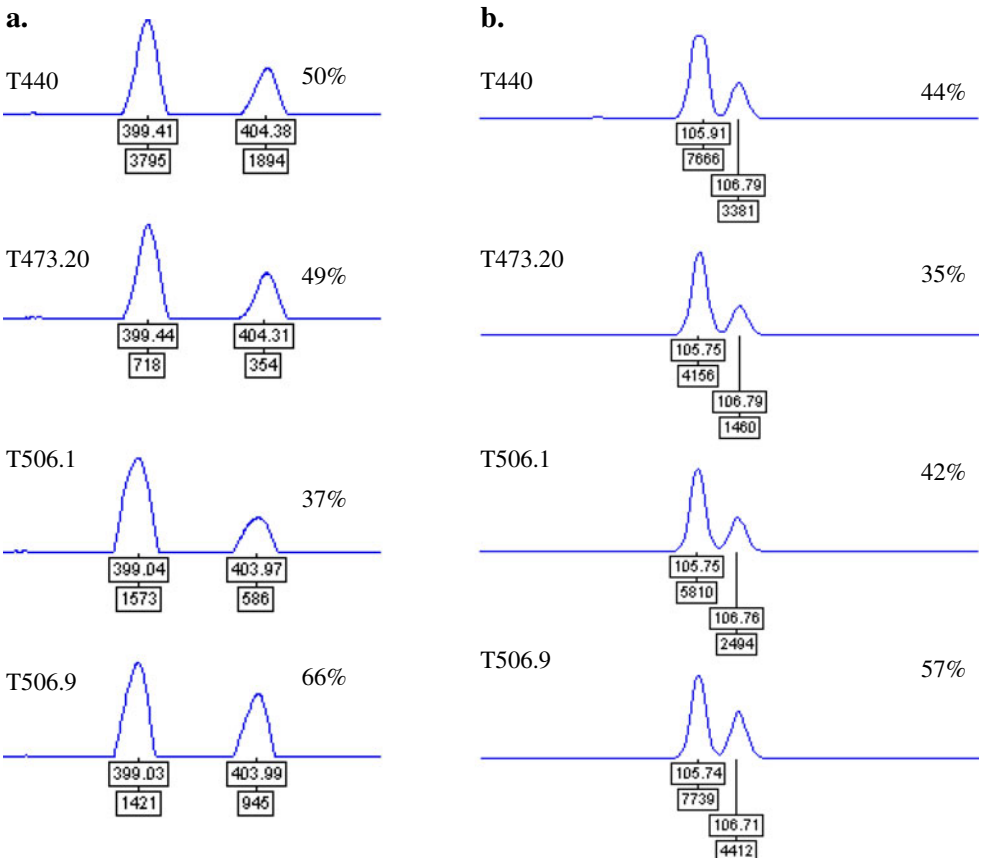

Fig. 5 **a** LOH of four neurofibromas (T440, T473.20, T506.1, and T506.9) at *TP53 Alu1*. **b** LOH in four neurofibromas (T440, T473.20, T506.1, and T506.9) at exon 6 of the *TP53* gene. All percentages refer to the level of LOH of the second allele

either direct or inverted repeats or both. In addition, for 13 of these mutations short (≥5 bp) purine or pyrimidine tracts were found within ±20 bp of the mutation. Such purine/pyrimidine tracts are known to promote genetic instability through formation of non-B DNA structures [46]. The remaining four micro-deletions and one micro-insertion were apparently mediated by direct/inverted repeat or symmetric elements. Only two (22%) somatic mutations in the second patient were single basepair substitutions whereas the majority of them were microlesions. Purine and pyrimidine tracts were found downstream and upstream of mutations E31:c.5888 A>C and E34:c.6478 A>G, respectively. All four deletions in the second patient were deduplications [47] mediated by inverted repeats. Two micro-insertions (E16:c.2451insG and E12b:c.1884insA) were mediated by inverted repeats and inversion E22:c.3806insC was mediated by direct repeat. Somatic mutations found in the third patient do not differ dramatically from the second patient; they are all mediated by either direct or inverted repeats. In addition, short alternating purine–pyrimidine tracts with Z-DNA-forming potential were found in the vicinity of six mutations. Motif complement to the

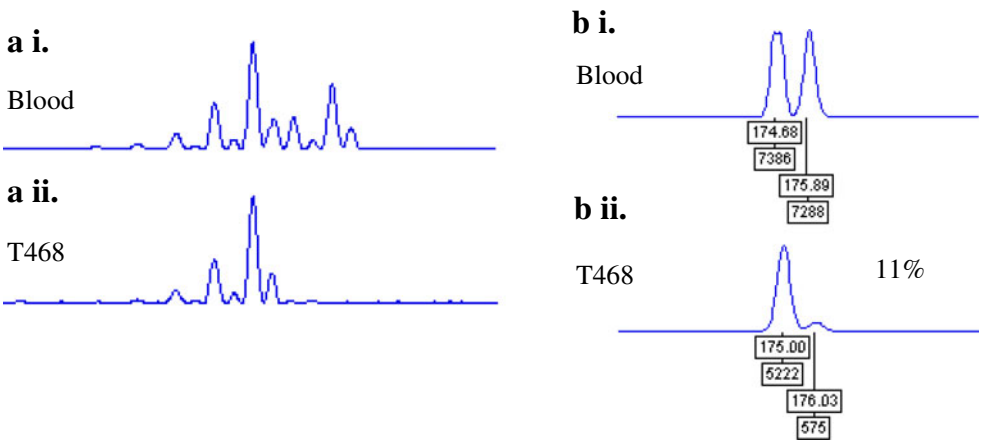

Fig. 6 **a** (*i*) Analysis of matched lymphocyte DNA with marker *D13S118*. **a** (*ii*) LOH of the *RB1* gene at *D13S118* in tumor T468. **b** (*i*) Analysis of matched lymphocyte DNA with marker *D13S917*; **b** (*ii*) LOH of the *RB1* gene at *D13S917* in tumor T468. All percentages refer to the level of LOH of the second allele

deletion hotspot, TGRRKM, was found in the vicinity of the remaining deletion E34:c.6364del114bp.

Discussion

This study has characterized the germline and somatic mutational spectrum in NF1 patients with high neurofibroma burden. Our main aim was to ascertain whether the neurofibromas in each patient developed independently, or were clonally derived. We also set out to determine whether a different molecular mechanism of tumourigenesis might underlie their development.

Three unrelated NF1 patients, all with a high burden of cutaneous neurofibromas, >550 and additional complications (Table 1) were recruited for this study. We demonstrated that each somatic mutation identified was the result of an independent event, clearly indicating that although some of these tumors had developed in very close proximity to each other, none of them were clonally derived (Supplementary Table 1). Indeed even in the situation where five tumors from within the same capsule were analyzed, only two exhibited LOH (Figs. 2 and 3), thus indicating that they are not all clonal. Twenty-eight of the somatic *NF1* mutations identified are also novel sequence changes, significantly contributing to the expansion of the *NF1* somatic mutational spectrum.

Although the somatic mutation detection rate for this cohort of neurofibromas is relatively low (64%), it is comparable with previous studies [11]. The problem of cellular heterogeneity is likely to contribute to the low mutation detection rate and a significant improvement in mutation detection may result by selectively enriching Schwann cells from tumors as shown by Maertens et al [48] or with the use of laser capture microdissection to specifically target mutated cells [49]. The undetected somatic mutations could also be located in the regions not screened in this study including deep intronic areas, the 5'and 3' UTRs as well as the *NF1* promoter region [50–52].

Bioinformatic analysis shows that the vast majority of mutations were mediated by either direct or inverted repeats. There are also differences in the inter-individual *NF1* somatic mutational spectrum identified in the three patients studied (Fig. 4). A comparison of the germline and somatic *NF1* mutations detected in these three patients failed to reveal any obvious correlation between the genotype and phenotype of the patients although the number is small hence a study of a larger patient cohort is warranted.

Overall, LOH was detected in 25% of tumors, in agreement with previous findings where LOH has been detected in ~10–30% of samples [12, 13, 30]. Previous studies by Däschner et al [53] and John et al [54] found little evidence for LOH, a similar finding to the third patient in our study. Low levels of LOH detection and inter-individual variability in the level of LOH could also be accounted for by cellular heterogeneity and the methodology used for analysis by different studies. In this study, no genomic deletions were found by MLPA in tumor DNA exhibiting LOH, indicating that mitotic recombination is the likely mutational mechanism in these tumors [55].

In view of the large number of cutaneous neurofibromas in each of these patients, we also analysed other potential modifying loci (*TP53*, *RB1*, and *CDKN2A*) which have been found to contribute to the development of plexiform neurofibromas [23, 24] and MPNSTs [19–22]. It was found that one tumor exhibited LOH which encompassed a large area of approximately 1 Mb at markers flanking *RB1*. Another four tumors exhibited *TP53* LOH at markers within the gene (Figs. 5 and 6). These five tumors were not found adjacent to any other tumor suggesting that there was no relationship between the anatomic location of the tumors and the development of additional somatic mutations. The flanking LOH markers for *TP53* at 17p13.2 and 17p12 did not show LOH indicating that LOH is restricted to the *TP53* gene. No detectable *TP53* deletions were identified by MLPA, suggesting mitotic recombination as a possible mutational mechanism. LOH could still be present in the remaining tumors in which no LOH of *TP53* and *RB1* was identified due to uninformative markers in these samples.

The global somatic mutational spectrum has been thought to be important for elucidating variable clinical expression. While benign and malignant NF1-associated tumors exhibit inactivation of both *NF1* alleles, the accumulation of additional somatic abnormalities represents a possible step towards malignant transformation. Therefore, identification of LOH at *TP53* and *RB1* loci, in addition to somatic *NF1* mutations in these benign tumors is an important finding as such somatic mutations are normally only associated with malignant NF1 tumors and cutaneous neurofibromas are not known to develop into MPNSTs [19–22]. If these genetic aberrations occur later during tumor formation then it is possible that these mutations represent a step towards malignant progression in these tumors. Additional somatic mutations involving genes from multiple genetic pathways could therefore indicate that in these patients, the occurrence of high tumor burden may be due to somatic mutations of other modifying loci, in addition to their *NF1* mutations.

In the current study, genome wide MSI was detected in 24% of the neurofibromas (21/89) but no sequence changes of the MMR genes were identified. The significance of MSI in these tumors is unclear as the level of MSI reported here is similar to previous findings in neurofibromas and is lower than has been identified in MPNSTs [11–15]. Patient

2 exhibited the highest level of MSI and had an MPNST. Patient 3, however, also had a malignant complication of B-CLL but had a significantly reduced level of MSI which was similar to that seen in patient 1 who was without malignant abnormalities (Table 1). The correlation of MSI and additional malignant complications is therefore unclear and would warrant a much larger study to determine any relationship. MSI in these neurofibromas is therefore more likely to represent generalized genomic instability rather than evidence of specific defects in the MMR genes. Copy number abnormalities, sequence changes in the other MMR genes or alterations to the methylation status of the MMR genes could also still account for the presence of MSI. Due to insufficient tumor tissue, immunohistochemical analysis of MMR proteins was not possible in these tumor samples.

The biological mechanisms which underlie the development of cutaneous neurofibromas in NF1 are still relatively unknown. Recent advances including the identification of SKPs as the cells thought to initiate neurofibroma formation will hopefully enhance our understanding of NF1 tumourigenesis, but there is currently insufficient information about their biological function [28]. Consequently, many aspects of neurofibroma development remain unexplained. Advancement in our understanding of the genes and cell types involved in neurofibroma formation will therefore underpin improvements in our knowledge of NF1 tumourigenesis. This study has demonstrated that despite some of the tumors being located adjacent to each other, they are not clonally derived. Perhaps of more significance, is the identification of LOH at *TP53* and *RB1* loci in these tumors. This is a significant finding in benign neurofibromas as such *TP53* and *RB1* LOH has only previously been found to be associated with malignant MPNSTs. These novel findings, therefore demonstrate for the first time, that the development of neurofibromas in NF1 patients with high tumor burden potentially differs from that of discrete neurofibromas studied in classical NF1 patients.

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The authors declare that they have no conflict of interest.

We declare that the experiments comply with the current laws of the United Kingdom.

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