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NF1 Mutations are Common in Desmoplastic Melanoma

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Abstract

Desmoplastic melanoma (DM) is a rare variant of melanoma with distinct clinical, histopathologic, and immunohistochemical features. Clinically, DM differs from conventional melanoma by a higher propensity for local recurrence and less frequent metastatic spread to regional lymph nodes. In its pure form, DM has a distinct appearance displaying a low density of fusiform melanocytes in a collagen-rich matrix. While a number of mutations have been identified in primary melanoma, including *BRAF*, *NRAS*, *GNAQ*, *GNA11* and *KIT*, and the occurrence of these mutations has been found to correlate to some extent with the histopathologic features, anatomic site and/or mode of sun exposure, no distinct set of mutations has so far been reported for DM. To study the potential association of neurofibromin (*NF1)* mutations with DM, we examined 15 desmoplastic and 20 non-desmoplastic melanomas by next-generation sequencing. Mutations of the *NF1* gene were found in 14 of 15 (93%) desmoplastic and 4 of 20 (20%) non-desmoplastic melanomas. The high frequency of *NF1* mutations in desmoplastic melanomas suggests an important role for NF1 in the biology of this type of melanoma.

Keywords

desmoplastic melanoma; neurofibromin 1; mutation; next-generation sequencing

INTRODUCTION

Desmoplastic melanoma (DM) is a rare variant of primary melanoma with distinct clinical, histopathologic and immunohistochemical features[1–4]. It tends to affect the head and neck region of elderly individuals[1, 3], but may occur anywhere, even in the young[5]. In its clinical

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course DM differs from other melanomas by a higher propensity for persistent growth/local recurrence[4, 6]. DM only rarely metastasizes to regional lymph nodes[7]. DM has been classified into pure and mixed variants[8–11]. Among patients with thick tumors, those with pure DM have a more favorable prognosis[8–10]. Histopathologically, the tumor cells of DM are usually fusiform and amelanotic. In its pure form, the tumor cells are dispersed at a low cell density in a densely collagenous matrix. Immunohistochemically, the tumor cells of DM often fail to react with antibodies to melan-A/MART-1, microphthalmia-associated transcription factor (MITF), tyrosinase, or gp100[12], but are usually positive for S100 protein, nerve growth factor receptor (NGFR), and/or SOX10[12–15].

Little is known about genetic changes in DM. While a number of mutations have been identified in melanoma, in particular mutations in *BRAF* (codon V600), *NRAS*, *GNAQ*, *GNA11*, and *KIT*, they are usually absent in DM. In a recent next-generation sequence analysis of a desmoplastic melanoma with sarcomatoid de-differentiation, we found inactivating mutations of neurofibromin 1 (*NF1*)[16]. A subset of desmoplastic melanomas were previously found to be associated with loss of *NF1* locus[17]. Recently, a patient with neurofibromatosis type 1 was found to have developed a DM[18]. Since *NF1* mutations are commonly found in peripheral nerve sheath tumors[19, 20], and given the morphologic overlap of such tumors with DM, we reasoned that *NF1* mutations may also be associated with DM, and therefore, examined DMs for their mutation status and compared them to a control set of primary and metastatic melanomas without desmoplasia.

MATERIALS AND METHODS

Patients

The study was approved by the institution's IRB. Fifteen primary desmoplastic and 20 non-desmoplastic melanomas (2 primary, 18 metastatic tumor samples) were randomly selected. Of the 15 DMs, 7 tumors were pure DM characterized by a pauci-cellular fibrosing malignant spindle cell proliferation. Eight tumors were combined or mixed DM with both a classic pauci-cellular as well as a solid spindle cell tumor component (Table 1). One of the tumors was previously published as a case report[16]. The non-desmoplastic melanomas included 10 superficial spreading melanomas, 5 nodular, 3 acral and 2 lentigo maligna melanomas.

Immunohistochemical Analysis

Five micron thick sections were taken from formalin-fixed, paraffin-embedded tissue, and stained with an automated immunohistochemistry system according to the manufacturer's guidelines (Ventana BenchMark XT, Tucson, AZ) using a standard avidin-biotin procedure and antibodies for S100 protein and Sox10.

DNA extraction and targeted sequencing

To enrich for the tumor cell population, non-tumor tissue was manually removed from formalin-fixed, paraffin-embedded tissue sections, and areas with at least 50% neoplastic cells were scraped into sterile Eppendorf tubes. DNA was extracted with a QIAamp DNA FFPE Tissue Kit (Qiagen) according to the manufacturer's instructions. The extracted DNA

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was subjected to deep-coverage targeted sequencing using an assay termed MSK-IMPACT (Memorial Sloan Kettering - Integrated Mutation Profiling of Actionable Cancer Targets).

The MSK-IMPACT assay uses target-specific oligonucleotides (NimbleGen SeqCap) to capture all protein-coding exons and selected introns of 341 cancer-associated genes (oncogenes, tumor suppressor genes, and components of pathways deemed actionable by targeted therapies) and was previously described[21]. Briefly, 250 ng genomic tumor DNA and a negative control pool of 10 "normal" blood DNAs was fragmented (Covaris E220), and used to prepare barcoded sequencing libraries (New England Biolabs, Kapa Biosystems). The sequencing libraries were pooled at equimolar concentrations (100 ng per library) and used in the capture reaction (NimbleGen SeqCap). To prevent off-target hybridization, we spiked in a pool of blocker oligonucleotides complementary to the full sequences of all barcoded adaptors. The captured libraries were sequenced on an Illumina HiSeq 2500 to generate 75 bp paired-end reads producing an average coverage of >500 per tumor.

Sequence data were de-multiplexed using CASAVA, and aligned to hg19 using BWA[22]. Local realignment and quality score recalibration were performed using the Genome Analysis Toolkit (GATK) according to GATK best practices[23]. Sequence data were analyzed to identify single nucleotide variants, small insertions/deletions (indels) using MuTect [24] and GATK, respectively. Single-nucleotide variants and indels were called if the variant allele frequency in the tumor was present in > 5% of sequencing reads, and if the variant allele in the tumor was > 5 times higher than in the negative control. We excluded variant alleles if they were present in more than 2% of reads in the negative control, and if they were previously reported in dpSNP[25] or the 1000 genome project[26]. Single-nucleotide variants and indels were reviewed manually using the Integrative Genomics Viewer[27]. Oncoprints and MutationMaps were generated in the cBio portal[28].

RESULTS

Clinical Characteristics

Fifteen primary cutaneous DM were analyzed. The clinical and pathologic findings are summarized in Table 1. The ages of the patients ranged from 24 – 85 (mean = 65.7 years, median = 67 years). They included 10 men and 5 women. Thirteen tumors occurred in sun-damaged skin of elderly individuals (Fig. 1). Two tumors occurred in skin without evidence of sun-damage in young adults. Ten tumors were located in the head and neck region, three on the upper trunk and two on the upper extremities.

Pathologic and Immunohistochemical Features

Histopathologically, 12 of the 15 DM had associated melanoma in situ (Fig. 1). Tumor thickness ranged from 1.5 – 25 mm (mean = 9.6 mm; median = 9.5 mm). None of the tumors were ulcerated. Four invasive tumors were confined to the dermis. Eleven tumors extended into the subcutis (Clark V).

The DM were strongly positive for S100 protein and SOX10, but negative for melan-A in the invasive tumor component. Melan-A was expressed by intraepidermal and intrafollicular melanoma cells (melanoma in situ).

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Molecular Findings

Shared mutations, which were present in at least 30% of tumors, are shown in Figure 2. The gene most commonly mutated in DM was *NF1*. Mutations of *NF1* were detected in 14 of 15 (93%) of primary cutaneous DM. The vast majority of mutations in *NF1* were nonsense, frameshift, or splice-site mutations that usually result in truncated non-functional proteins (Fig. 3). Genes mutated in at least 50% of the tumors included protein tyrosine phosphatase, receptor type, T (*PTPRT*), protein tyrosine phosphatase, receptor type, D (*PTPRD*), tumor protein p53 *(TP53)* and Ros protooncogene 1 (*ROS1*).

Mutation in *BRAF*, *GNAQ*, *GNA11* and *KIT* were not identified in our cohort of DM. Only one tumor (case #7) had a *NRAS* mutation. Interestingly, this tumor harbored two missense mutations in *NF1*, whereas most other DM contained at least one deleterious *NF1* mutation (splice-site, non-sense, or frameshift mutations).

NF1 Mutations in Non-desmoplastic Melanoma

To assess the frequency of *NF1* mutations in non-desmoplastic cutaneous melanomas, we analyzed 20 tumors (18 metastases from known non-desmoplastic primaries, 2 primary melanomas). The tumors included 10 superficial spreading melanomas, 5 nodular melanomas, 3 acral melanomas and 2 lentigo maligna melanomas. *NF1* mutations were detected in 4 tumors (one lentigo maligna melanoma and 3 nodular melanomas) (Table 2). Three of the four non-desmoplastic melanomas with *NF1* mutations originated from the skin of the head neck region; one tumor was from the skin of the back. In all non-desmoplastic melanomas of this series, *NF1* was associated with another mutation of the RAS/RAF family: two cases had *NRAS* and another two had *BRAF* mutations.

DISCUSSION

We had previously noted inactivating mutations of *NF1* in a combined tumor of desmoplastic and dedifferentiated sarcomatoid melanoma[16]. We now report that *NF1* mutations are not unique to that case, but occur at high frequency in DM: *NF1* mutations were found in 14 of 15 (93%) of DM.

In our control group of 20 cutaneous melanomas without desmoplasia, *NF1* mutations were detected in 4 (20%) cases. Prior studies on the mutation profile of melanomas reported a frequency of *NF1* mutations in 16 of 121 (13%) tumors[29], and in 13 of 91 (14%)[30], respectively. In a recent review the overall frequency of *NF1* mutations was estimated at 14% of cutaneous melanomas, with a total of 475 specimens analyzed[31]. No information was provided about the histopathologic subtype, but it is unlikely that a significant number of DM was included in those studies, because DM is a rare variant of melanoma (approximately 4 % of primary melanomas)[3, 32]. Thus, compared to those reports and our control group of non-desmoplastic melanomas, *NF1* mutations are more commonly found in DM.

NF1 encodes neurofibromin 1, a negative regulator of the Ras/mitogen-activated protein kinase (MAPK) pathway, a key pathway in melanomagenesis[31]. NF1 is a Ras-specific GTPase-activating protein that accelerates the hydrolysis of the active GTP-bound form of

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Ras into its inactive GDP-bound form[33], but this is not the only mechanism, by which NF1 can downregulate Ras[31]. Somatic mutations in *NF1* have been in a variety of cancers, including melanoma[31]. Loss of NF1 detected in melanoma was reported in cell lines in the early 1990s [34, 35], and one study reported loss of *NF1* locus in a subset of DMs[17]. Recently, loss of NF1 was shown to prevent BRAF oncogene-induced senescence in melanoma[36]. In addition, one of the mechanisms of resistance for RAF-inhibitors is thought to occur via loss of NF1 expression[37].

NF1 mutations and loss of NF1 protein expression are known to occur in over 90% of neurofibromatosis 1–associated and in over 40% of sporadic malignant peripheral nerve sheath tumors (MPNST)[19, 20, 38]. MPNST and DM may show considerable morphologic and immunohistochemial overlap. The tumors reported herein are unequivocal melanomas and not MPNSTs, since all but three DM of this series were associated with melanoma in situ, and the invasive component of all tumors was centered in the dermis and showed strong and diffuse labeling for S100 protein and Sox10. Additionally, none of our patients had a history or clinical signs of neurofibromatosis.

A number of additional genes were commonly mutated in the DM of this series (Fig. 2). These are genes known to be mutated in melanoma, but compared to their reported frequency in prior studies[29, 30] they seem to be more common in DM. *TP53* mutations, for example, were detected in 19% of melanomas by Hodis et al[29] and in 12% of tumors by Krauthammer et al[30]. In our series of DM, *TP53* was mutated in 60% of cases (Fig. 2) and interestingly, the majority of *TP53* alterations were missense mutations that had clustered in the DNA binding domain.

Other highly mutated genes included the protein tyrosine phosphatase, receptor T (*PTPRT*, 67%) and D *(PTPRD*, 60%), which are known as tumor suppressor genes and recognized for their regulatory function on numerous cellular processes including cell growth, cell fate, and cell cycle. Although, the high frequency of mutations in these genes compared to prior sequencing studies of unselected melanoma (Hodis et al[29]: *PTPRT: 26%, PTPRD: 17%;* Krauthammer et al[30]: *PTPRT: 0%, PTPRD: 24%)* suggests some oncogenic impact in DM, thorough functional studies will be necessary to prove their pathogenetic relevance for several reasons: First, melanoma in general has a high background mutation rate, and it is difficult to distinguish pathogenetic relevant driver-mutations from irrelevant passenger-mutations. Previous studies reported very high point mutation rates in patients with a history of chronic sun exposure, and given the association of DM with chronic UV exposure[39], high mutation rates in DM should come as no surprise. Second, whereas most mutations in *NF1* were deleterious (nonsense, frameshift, or splice-site mutations), the mutation pattern found in the other genes was mainly composed of missense mutations. Missense mutations usually result in the exchange of one amino acid, and thus may have only minor effects on the protein function. Third, the missense mutations were quite randomly distributed across the genes and no hotspot mutations were found. Consequently, the impact of missense mutations on the protein function is unclear, and functional studies are necessary to analyze their oncogenic consequences.

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In summary, having used targeted next-generation sequencing for 341 cancer related genes, we document that inactivating *NF1* mutations are more common in DM than in non-desmoplastic melanomas. The finding provides further evidence that DM is, at the molecular level, distinct from conventional melanoma. The fact that *NF1* mutations are commonly seen in both pure and mixed DM suggests that they occur early in the evolution of DM and do not merely reflect tumor progression. This is further supported by the fact that *NF1* mutations can be found in a lentigo maligna melanoma in situ (KJB, unpublished observation). It is possible that mutations in genes that are not covered by our sequencing assay may also be associated with and play an important role in DM. However, given the high frequency of *NF1* mutations in our series of tumors, we believe that NF1 is relevant for the biology of DM and for discussions of targeted treatment of recurrences thereof.

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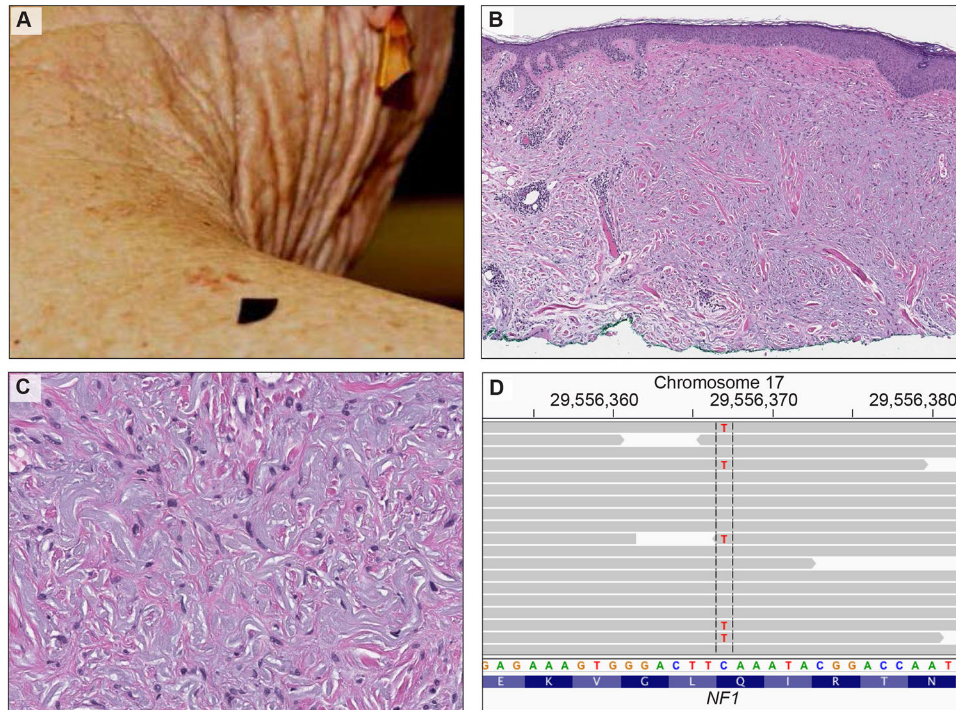

Figure 1.

Clinical, histological, and genetic features of desmoplastic melanoma. **A**, clinical picture of an 81-year-old woman with an irregular, brown-reddish skin lesion on her right shoulder. **B**, Histopathology of desmoplastic melanoma: Melanoma in situ (atypical proliferation of solitary units and nests of melanocytes at the dermoepidermal junction) is associated with an infiltrative pauci-cellular fibrosing spindle cell proliferation in the dermis with focal lymphocytic aggregates. **C**, The dermal spindle cells are dispersed as individual units (not aggregated in sheets or fascicles). They have hyperchromatic nuclei. **D**, The targeted sequencing data displayed in the Integrative Genomics Viewer (IGV). The gray arrows illustrate the individual sequencing reads aligned to exon 21 of the *NF1* gene. The cytosine at base pair position 2734 was replaced by thymine (c.2734C>T) leading to a premature stopcodon (p.Q912*) and to a truncated NF1 protein.

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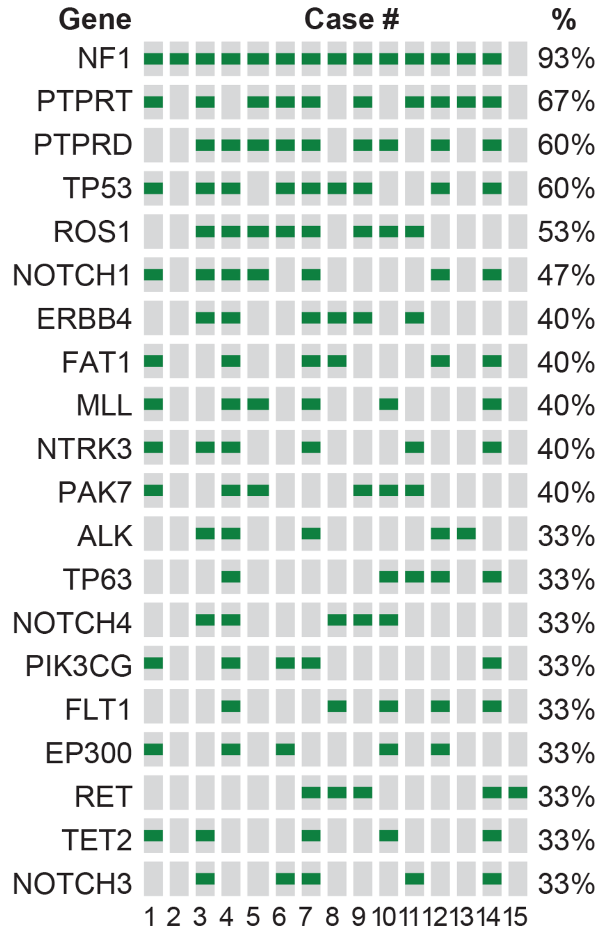

Figure 2.

Mutations detected by targeted sequencing and shared by at least 30% of desmoplastic melanomas. The columns denote the samples (according to Table 1), the rows refer to the genes, and the green squares indicate mutations.

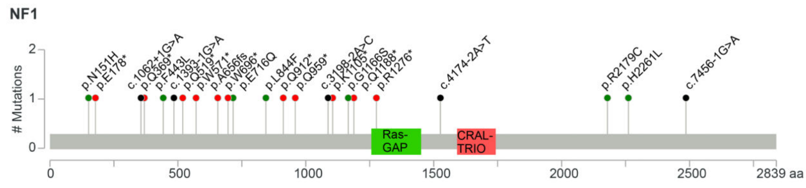

Figure 3.

Mutation pattern in NF1. The y-axis refers to the number of mutations and the x-axis to the amino acids. NF1 contains a Ras-GAP domain (GTPase-activator protein for Ras-like GTPase) between amino acids 1256–1451, and a CRAL-TRIO domain between amino acids 1591–1736. Missense mutations are displayed in green, nonsense mutations in red, and splice-site mutations in black. Missense and nonsense mutations are annotated to the protein level. Splice-site mutations are referenced to the coding DNA sequence.

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Table 1

Desmoplastic melanoma – clinical findings, pathologic features, and *NF1* mutation status.

Case #	Age, Sex	Site	MIS	Thickness	Clark Level	DM Type	*NF1* Mutation		
							Mutation Type	cDNA	Protein
1	82M	Scalp	Yes	1.9 mm	V	Pure	Nonsense	c.3562C>T	p.Q1188*
2	24M	Arm	Yes	9.5 mm	V	Pure	Nonsense	c.2875C>T	p.Q959*
3	85F	Face	Yes	7.2 mm	V	Pure	Nonsense	c.1555C>T	p.Q519*
							Splicesite	c.7456-1G>A	p.? (splice)
4	81F	Shoulder	Yes	1.5 mm	IV	Pure	Missense	c.2530C>T	p.L844F
							Nonsense	c.2734C>T	p.Q912*
5	77F	Face	Yes	2.5 mm	IV	Pure	Nonsense	c.1105C>T	p.Q369*
							Missense	c.2146G>C	p.E716Q
6	66M	Scalp	Yes	7 mm	V	Pure	Missense	c.6472C>T	p.R2179C
							Nonsense	c.1713G>A	p.W571*
							Missense	c.3496G>A	p.G1166S
7	76M	Scalp	Yes	1.8 mm	V	Mixed	Missense	c.451A>C	p.N151H
							Missense	c.6782A>T	p.H2261L
8	85M	Neck	No	18 mm	V	Mixed	Nonsense	c.3313A>T	p.K1105*
							Nonsense	c.3826C>T	p.R1276*
9	82M	Scalp	Yes	16 mm	V	Mixed	Splicesite	c.3198-2A>C	p.? (splice)
10	67M	Face	Yes	3 mm	IV	Mixed	Nonsense	c.532G>T	p.E178*
							Frameshift	c.1968_1969delCT	p.A656fs
11	58M	Shoulder	No	11.5 mm	V	Mixed	Splicesite	c.1062+1G>A	p.? (splice)
12	66M	Scalp	Yes	15 mm	V	Mixed	Splicesite	c.4174-2A>T	p.? (splice)
13	51M	Chest	Yes	9.5 mm	V	Mixed	Missense	c.1327T>C	p.F443L

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Case #	Age, Sex	Site	MIS	Thickness	Clark Level	DM Type	*NF1* Mutation		
							Mutation Type	cDNA	Protein
14	49F	Nose	Yes	15 mm	V	Mixed	Splicesite	c.1393-1G>A	p.? (splice)
							Nonsense	c.2088G>A	p.W696*
15	37F	Trunk	No	25 mm	V	Pure	-	-	-

Abbreviations: M = male; F = Female; MIS = melanoma in situ; mm = millimeter; DM = desmoplastic melanoma; NF = neurofibromin

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Table 2

NF1 mutations in 4 of 20 non-desmoplastic melanomas.

Case #	Age, Sex	Site	Thickness	Melanoma Type	*NF1* Mutation	
					cDNA	**AAchange**
1	68 M	Neck	2.2 mm	Lentigo Maligna	c.C1145T	S382F
					c.1499_1521delTTCATGCAGATCCAAAGCTCTTG	I500fs
					c.C1997T	S666F
					c.C7126T	H2376Y
					c.C20T	S7F
2	84M	Ear	5 mm	Nodular	c.C3097T	Q1033X
					c.C3652T	Q1218X
3	60M	Scalp	6.5 mm	Nodular	c.T3239A	L1080Q
4	62M	Back	9.6 mm	Nodular	c.C3028T	Q1010X
					c.4321delT	L1441X

Abbreviations: M = male; F = female; mm = millimeter; NF1 = neurofibromin; AA = amino acid