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Multiple Congenital Melanocytic Nevi and Neurocutaneous Melanosis Are Caused by Postzygotic Mutations in Codon 61 of *NRAS*

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Congenital melanocytic nevi (CMN) can be associated with neurological abnormalities and an increased risk of melanoma. Mutations in *NRAS*, *BRAF*, and *Tp53* have been described in individual CMN samples; however, their role in the pathogenesis of multiple CMN within the same subject and development of associated features has not been clear. We hypothesized that a single postzygotic mutation in *NRAS* could be responsible for multiple CMN in the same individual, as well as for melanocytic and nonmelanocytic central nervous system (CNS) lesions. From 15 patients, 55 samples with multiple CMN were sequenced after site-directed mutagenesis and enzymatic digestion of the wild-type allele. Oncogenic missense mutations in codon 61 of *NRAS* were found in affected neurological and cutaneous tissues of 12 out of 15 patients, but were absent from unaffected tissues and blood, consistent with *NRAS* mutation mosaicism. In 10 patients, the mutation was consistently c.181C>A, p.Q61K, and in 2 patients c.182A>G, p.Q61R. All 11 non-melanocytic and melanocytic CNS samples from 5 patients were mutation positive, despite *NRAS* rarely being reported as mutated in CNS tumors. Loss of heterozygosity was associated with the onset of melanoma in two cases, implying a multistep progression to malignancy. These results suggest that single postzygotic *NRAS* mutations are responsible for multiple CMN and associated neurological lesions in the majority of cases.

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INTRODUCTION

Congenital melanocytic nevi (CMN) can cover up to 80% of the body surface area and large CMN, which occur in 1 in 20,000 births (Castilla *et al.*, 1981), are usually associated with multiple smaller nevi (Figure 1). CMN can be associated with neurological abnormalities, sometimes termed neurocutaneous melanosis, although many of the abnormalities are not melanocytic. The commonest finding is foci of melanin-producing cells within the brain parenchyma, found on magnetic resonance imaging in ~20% of affected children. Other neurological associations comprise communicating hydrocephalus, arachnoid cysts, syringomyelia, tumors (including astrocytoma, choroid plexus papilloma, ependymoma, and pineal germinoma), and malformations such as Dandy–Walker or Arnold–Chiari (Frieden *et al.*, 1994; Foster *et al.*, 2001; Agero *et al.*, 2005; Kinsler *et al.*, 2008; Ramaswamy *et al.*, 2012). Leptomeningeal melanocytosis is a diagnosis that was previously made only at post-mortem but now can be made radiologically, and is a description of leptomeningeal deposits with a characteristic signal for melanin in a discrete or diffuse pattern. These particular lesions can be stable and benign in behavior, but are frequently rapidly progressive and capable of metastasis. Histology is not always informative, and clinical and radiological progressions are the best indicators of prognosis currently available. Neurological symptoms in patients with CMN can be present without radiological abnormality (Ruiz-Maldonado *et al.*, 1997), and this disconnect is likely to be due to developmental intraparenchymal lesions below the resolution of

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Abbreviations: CGH, comparative genomic hybridization; CMN, congenital melanocytic nevi; CNS, central nervous system; MC1R, melanocortin 1 receptor

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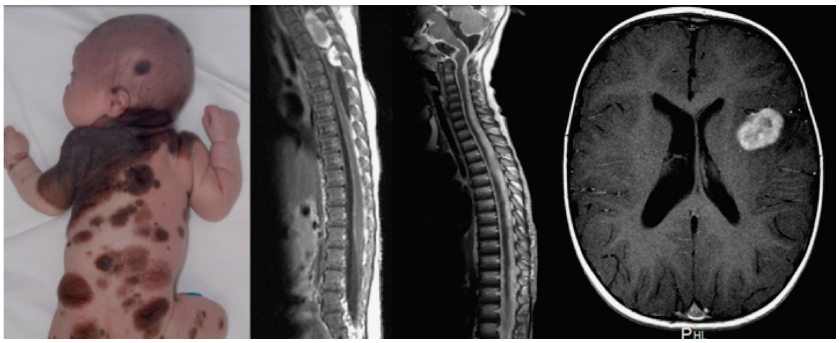

Figure 1. Clinical and radiological images of congenital melanocytic nevus (CMN) syndrome. (From left to right) An example of the cutaneous phenotype of multiple CMNs (written consent for publication was obtained); magnetic resonance (MR) images showing two thoracic spinal tumors (neurocristic hamartomata), diffuse leptomeningeal melanocytosis, and frontal lobe meningioma.

current imaging (Kinsler *et al.*, 2012c). Overall, the prevalence of radiological neurological abnormalities increases with the severity of cutaneous phenotype (i.e., size of the largest CMN and total number of nevi), and in males (Kadonaga and Frieden, 1991; Kinsler, 2008; Ramaswamy *et al.*, 2012).

In addition to the developmental abnormalities, CMN are a known risk factor for malignant melanoma in postnatal life. The absolute risk is associated with the severity of the cutaneous phenotype, estimated as a 1–2% lifetime risk for all individuals with CMN, but rising to 10–15% in those with the largest nevi. For those with severe cutaneous phenotypes, the risk peaks in childhood, at an age when melanoma is otherwise extremely rare (Krengel *et al.*, 2006). Importantly, in cases where melanoma does arise, the primary tumor is not necessarily within the skin, but often within the central nervous system (CNS), and occasionally elsewhere (Hale *et al.*, 2005; Krengel *et al.*, 2006; Kinsler *et al.*, 2009). Melanoma in these children is usually highly aggressive and refractory to therapy, being rapidly fatal in all cases that we have treated over the past 20 years (including unpublished cases), and in the vast majority of adequately documented cases reported in the literature (Krengel *et al.*, 2006; Kinsler *et al.*, 2009). The median age for the onset of melanoma in children with CMN is estimated at 7 years from a review of the literature (Krengel *et al.*, 2006).

The phenotype of multiple CMN does not follow a Mendelian pattern of inheritance with only a few familial cases reported (de Frieden and Williams, 1994; Wijn *et al.*, 2010). This, and the description of multiple CMN in one twin of a monozygotic pair (Amir *et al.*, 1982), led us to consider a causative somatic mutation for multiple CMN and associated neurological lesions. Mutations already described in single samples of CMN were deemed good candidates in this model, namely those in *NRAS* (Papp *et al.*, 1999; Bauer *et al.*, 2007; Dessars *et al.*, 2009; Phadke *et al.*, 2011; Wu *et al.*, 2011), *BRAF* (Papp *et al.*, 1999; Pollock *et al.*, 2003; Kumar *et al.*, 2004; Papp *et al.*, 2005; Ichii-Nakato *et al.*, 2006; Dessars *et al.*, 2007; Adjei *et al.*, 2008), and TP53 (Papp *et al.*, 1999), and polymorphisms in *melanocortin 1 receptor* (*MC1R*) (Papp *et al.*, 1999; Kinsler *et al.*, 2012b). We have previously reported a role for germline *MC1R* genotype in CMN, and had shown at that time consistency of germline and somatic genotype, dismissing *MC1R* as a somatic mosaic candidate (Kinsler *et al.*, 2012b). Of the other candidates, *NRAS* was the strongest for larger CMN, although mutation rates in studies of single samples were variable. Furthermore, *NRAS* mutations have been described in many other types of tumors (COSMIC, 2012), consistent with the 25% rate of *RAS* family mutations in all human tumors (Castellano and Downward, 2011). In an attempt to uncover a causal mutation, we therefore postulated that neurological tumors in patients with CMN may also harbor activating *NRAS* mutations, and that lesions from a single individual may have a consistent genotype with skin lesions as a consequence of a single neuroectodermal mutation in the developing embryo, leading to both neurological and cutaneous features. Consistent with this, our results demonstrate that different CMN lesions from patients with multiple CMN contain identical codon 61 *NRAS* mutations and that neurological lesions from these patients also contain mutations at codon 61 of *NRAS*.

RESULTS

NRAS mutations in CMN

The proportion of nevus cells in a biopsy of CMN differs between lesions, and failure to identify mutations in CMN could result from this mosaicism of nevus and non-nevus cells within the lesions. The percentage of *NRAS* codon 61 mutant alleles measured on direct sequencing in cutaneous lesions varied from 7 to 48%, which may reflect the proportion of nevus cells in the biopsy. Therefore, in order to improve the detection of *NRAS* mutation in the samples, *NRAS* was sequenced after a site-directed mutagenesis approach that allowed enzymatic digestion of the wild-type allele. Samples with percentages $<20\%$ were enzymatically digested, and the percentage of mutant alleles in those rose to 25–63% after a single cycle of digestion. Measurement of the percentage mosaicism before digestion was validated using samples of known percentage heterozygosity (Supplementary Figure S1 online), and accuracy was found to be high. Using this approach, *NRAS* codon 61 mutations were identified in the CMN of 10 of the 13 subjects whose cutaneous tissue was available for sequencing (Table 1), with c.181C>A, p.Q61K in 8 subjects and c.182A>G, p.Q61R in 2 subjects. The same *NRAS* codon 61 mutation was seen in

Table 1. Genotype of samples of 15 patients who provided tissue

	Tissue type							
Case number	Unaffected skin	CMN	Unaffected meninges	Diffuse leptomeningeal melanocytosis	Primary melanoma	Metastic melanoma	CNS tumor (non-melanocytic)	Blood
1		■■■		■			■	■
2	■	■■						■
3	■	■■						■
4		■						■
5		■■			■HZ	■HZ		■
6		■■ ■HZ						
7	■	■■						■
8		■■						
9		■■						■
10		■						■
11							■	■
12	■■■	■■■	■	■■■■■■	■	■		■
13							■	
14		■■						■
15	■	■						
Mutations	0/7	19/26	0/1	7/7	2/2	2/2	3/3	0/11

Key ■ = *NRAS* Q61R mutation, ■ = *NRAS* Q61K mutation, ■ = wild type at codon 61 *NRAS*, HZ = homozygous

Abbreviations: CMN, congenital melanocytic nevus; CNS, central nervous system.
Each symbol represents an anatomically separate lesion.

each of the anatomically separate CMN from the same subject, except for case 12 where one of three CMN failed to show a mutation.

NRAS mutations in neurological lesions from patients with CMN

All 10 neurological samples from 5 patients (or 11 from 5 patients if the sample of primary CNS melanoma is included), melanocytic and nonmelanocytic, were positive for the oncogenic *NRAS* missense mutation c.181C>A p.Q61K (Table 1). These included one choroid plexus papilloma, one neurocristic hamartoma, one meningioma, and two cases of leptomeningeal melanocytosis, where the same *NRAS* mutation was observed in six separate anatomical samples of affected meninges taken at post-mortem from one individual. No mutation was noted in the sample of normal meninges from this subject. In both cases where neurological tissues and CMN were available, the patients with CNS mutations also had the same mutation in affected skin. In order to determine whether the detection of mutations in the CMN and neurological samples were simply because of a germline *NRAS* mutation, DNA from blood was sequenced in 11 of the 15 subjects who provided tissue for the study, and in 42 other patients with large CMN. All 53 blood DNA samples showed wild-type *NRAS*, even after three cycles of PCR/enzymatic digestion, suggesting that the codon 61 mutations were restricted to affected tissues. In support of this, no mutations were detected in unaffected skin of two subjects who provided nonlesional skin samples and in whom *NRAS* alterations were noted in their CMN (Table 1).

NRAS mutations in melanoma from CMN subjects

Of the 15 patients who provided tissue samples, 3 died of melanoma, with the primary tumor in the skin (case 5), leptomeninges (case 6), and cerebellum (case 12). Primary melanoma samples were only available for DNA analysis from cases 5 and 12. In case 5, pre- and postmalignant samples were available from the same cutaneous lesion, which revealed progression from heterozygosity to homozygosity for the mutation Q61K with the onset of malignancy (Figure 2). The only other case in which homozygosity for *NRAS* mutation was found (in this instance Q61R) was from case 6 where two histologically benign, but clinically highly proliferative, CMN samples were analyzed. At 3 months after the homozygous cutaneous sample was excised, the patient developed leptomeningeal melanocytic disease, which was histologically and clinically indistinguishable from malignant melanoma, and was fatal after spreading to the abdomen via the ventriculoperitoneal shunt. In case 12 in which *NRAS* was mutated in the CMN, there was no loss of heterozygosity for *NRAS* mutations in the primary CNS melanoma sample. Melanoma samples from cases 5 and 12 were negative for *BRAF* V600E mutations.

Array comparative genomic hybridization (CGH) findings in the melanoma samples were of multiple losses and gains of parts of chromosomes (Figure 3), consistent with the previously described pattern of malignant melanoma arising within CMN (Bastian *et al.*, 2002). Notably, array CGH at 50 kb resolution in case 5 did not show a deletion encompassing *NRAS*, implying that the loss of heterozygosity was either due to an additional Q61K mutation in the normal allele or a very small deletion at this locus. In both cases 5 and

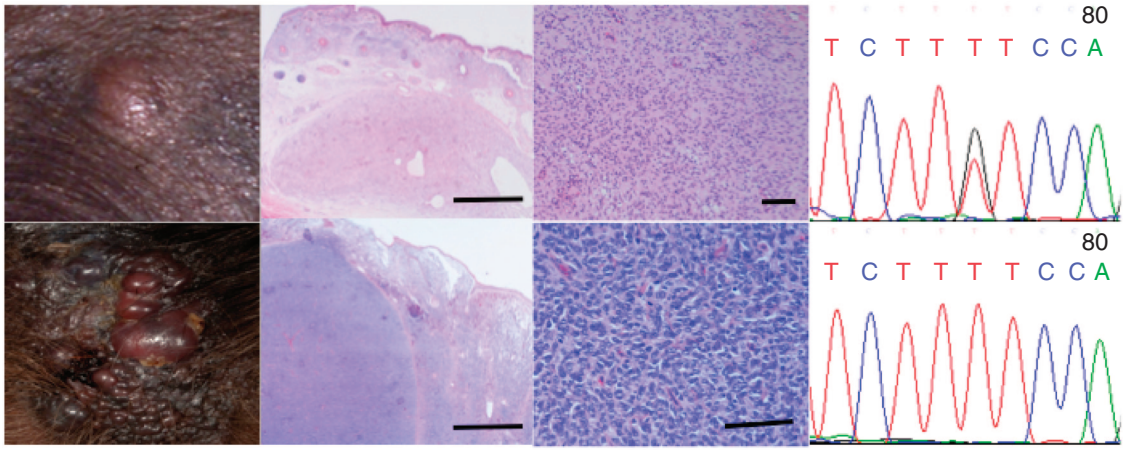

Figure 2. Progression from *NRAS* Q61K heterozygosity to homozygosity with the onset of malignancy. (Above) Congenital proliferative nodule within a congenital melanocytic nevus (CMN). (From left to right) Clinical appearance, hematoxylin and eosin (H&E)-stained section at ×20 magnification, DNA sequence chromatogram (reverse) showing heterozygous Q61K mutation. (Below) Malignant melanoma arising within the same CMN in the same patient 5 years later. (From left to right) Clinical appearance, H&E-stained section at ×20 magnification, DNA sequence chromatogram showing homozygous Q61K mutation.

a

Case number	Large copy number changes (>30Mb)	Coordinates (Hg19)
5	Loss 1p36.33-p34.3	chr1:567,050-37,543,869
	Gain 2q23.1-q35	chr2:149,315,277-215,940,078
	Loss 5q21.3-q35.3	chr5:105,186,889-180,699,371
	Loss chromosome 9	Whole chromosome
	Loss 11q14.3-q25	chr11:90,462,997-134,677,947
	Loss 12q22-q24.3	chr12:94,171,493-133,563,446
	Loss chromosome 17	Whole chromosome
12	Gain 1q21.1-q44	chr1:143,743,957-249,167,297
	Loss chromosome 3	Whole chromosome
	Loss 5p15.33-q11.2	chr5:379,496-57,783,740
	Gain 6p25.3-p11.2	chr6:175,687-57,037,799
	Gain chromosome 8	Whole chromosome
	Loss 9p24.3-p21.1	chr9:45,724-31,673,803
	Loss 11p15.5-p13	chr11:1,933,704-35,050,113
	18 loss	Whole chromosome

b

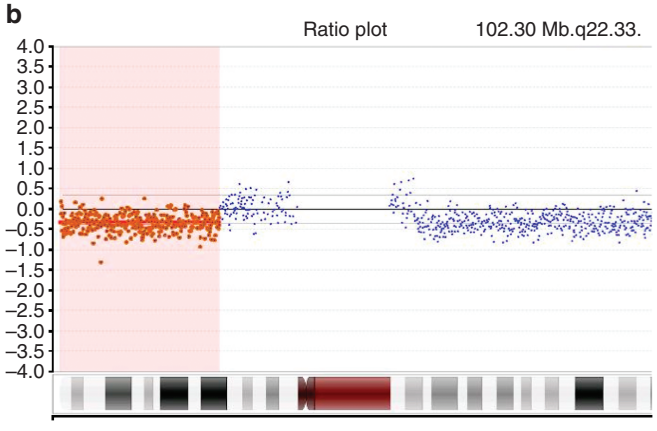

Figure 3. Array comparative genomic hybridization (CGH) in melanoma samples. Array CGH findings in both available primary melanoma samples (cases 5 and 12 from Table 2) showing (**a**) a list of all gains and losses of ⩾30 Mb, and (**b**) heterozygous partial deletion of chromosome 9p, including the *CDKN2A* locus.

12, array CGH revealed a deletion in chromosome 9p (del chr9:45,724-40,026,947 and del chr9:45,724-31,673,803, respectively), with both deletions including the *CDKN2A* locus (Figure 3). In comparison, the array CGH from the clinically stable diffuse leptomeningeal melanocytosis from case 1 did not show any large losses or gains.

DISCUSSION

As predicted by Happle (1987), somatic mosaicism for genes likely to be lethal in the germline has recently been found to be the cause of several conditions with severe clinical phenotypes involving the skin, including Proteus syndrome (Lindhurst *et al.*, 2011), CLOVES syndrome (Kurek *et al.*, 2012), and Schimmelpenning syndrome (Groesser *et al.*, 2012). Our results from multiple CMN and neurocutaneous melanosis indicate that a similar somatic mosaicism is responsible for the phenotypic abnormalities in this condition, and suggest that the mutation probably occurs in the developing neural crest or neuroectoderm, although the exact cell lineage is not yet clear. Indeed, in the context of only 0.77% of neurological tumors in online databases being positive for *NRAS* mutations (COSMIC, 2012), our findings of *NRAS* alterations in the neurological as well as the skin lesions is highly supportive of a unifying causal mutation in these patients that affects pigmentary cells in the skin and pigmentary and/or non-pigmentary cells in the CNS. Furthermore, patients with CNS mutations also had the same mutation in affected skin, and patients with multiple cutaneous lesions harbored the same mutation in each lesion, but not in non-lesional skin. As all blood samples were negative for *NRAS* mutations, we hypothesize that this mutation may be lethal in the germline. The absence of codon 61 mutations in any samples from 3 of the 15 patients suggests that mosaic mutations in another *NRAS* codon or in another gene are likely to be responsible in this minority of cases.

NRAS is an extensively characterized oncogene involved in the control of key cell signaling pathways (Castellano and Downward, 2011; Pylayeva-Gupta *et al.*, 2011). Transformation between inactive (guanosine diphosphate bound) and active (guanosine triphosphate bound) states allows RAS to act as a molecular switch (reviewed in Pylayeva-Gupta *et al.*, 2011), controlling the signaling of RAF and phosphatidylinositol 3-kinase, and thereby the activation of the RAF/MEK/ERK pathway and Akt, respectively. Codon 61 in the guanosine triphosphate–binding site is crucial for normal inactivation, and mutations at this site lead to constitutive activation of *NRAS*. Our findings of *NRAS* codon 61 mutations in multiple CMN are supported by evidence from animal models, where injection of EGFP-NRAS^{Q61K} fusion protein into developing zebrafish leads to multiple cutaneous nevi, and transgenic zebrafish overexpressing NRAS^{Q61K} in developing melanocytes showed widespread hyperpigmentation (Dovey *et al.*, 2009). A transgenic *NRAS*^{Q61K} murine model also developed CMN-like lesions (Ferguson *et al.*, 2010). Furthermore, comparison of our results with the recent report of *NRAS* codon 12 mutation mosaicism in two patients with juvenile myelomonocytic leukemia, with no neurological or cutaneous features (Doisaki *et al.*, 2012), suggests that the phenotype resulting from developmental mutations in *NRAS* are specific to the affected cell type and/or affected codon.

Germline mutations in the RAS/RAF/MEK/ERK pathway give rise to a group of conditions now termed RASopathies (Tidyman and Rauen, 2009). These are distinct but phenotypically related conditions including Neurofibromatosis type 1, Costello syndrome, Cardiofaciocutaneous syndrome, Noonan syndrome, and Leopard syndrome, most of which have a pigmentary component. Although the original description of RASopathy defined a germline genotypic abnormality (Tidyman and Rauen, 2009), our current findings and those in Schimmelpenning syndrome (Groesser *et al.*, 2012) suggest that ''mosaic RASopathies'' could also be recognized as part of this spectrum. A recent finding in children with CMN is characteristic facial features (Kinsler *et al.*, 2012a), namely wide or prominent forehead, apparent hypertelorism (the term used for hypertelorism described under the age of 15 years), eyebrow variants, periorbital fullness, small/short nose, narrow nasal ridge, broad nasal tip, broad or round face, full cheeks, prominent premaxilla, prominent/long philtrum, and everted lower lip. This finding has relevance as the neuroectoderm also contributes to the development of cartilage and bones of the face. The germline RASopathies all have characteristic facial features, demonstrating the effect of RAS/RAF/MEK/ERK pathway imbalance on facial development (Zenker, 2011). More specifically, germline mutations in other codons (i.e., not codon 61) of *NRAS,* such as those in a subset of Noonan syndrome, are known to affect facial development in humans (Cirstea *et al.*, 2010) and in zebrafish (Runtuwene *et al.*, 2011). Although speculative, it is feasible that the current finding of *NRAS* mutation mosaicism in individuals with multiple CMN could explain the facial similarities in this patient population as a result of a mutation in neuroectoderm cells affecting precursors involved in facial development.

An interesting additional question is how these somatic *NRAS* mutations are related to the recent finding of a higher frequency of two *MC1R* variant alleles in the germline of individuals with CMN, and a phenotype exacerbating the effect of certain alleles (Kinsler *et al.*, 2012b). Interactions between germline *MC1R* genotype and somatic mutations in the mitogen-activated protein kinase pathway (*BRAF/NRAS*) have been reported; however the data have been conflicting (Landi *et al.*, 2006; Hacker *et al.*, 2010; Scherer *et al.*, 2010). A possible explanation would be that reduced or altered signaling via *MC1R* could promote clonal growth of *NRAS* mutated cells, as has been reported for *p53* clonal patches (Robinson *et al.*, 2010), but further studies will be required to test this hypothesis.

In conclusion, our data suggest that multiple CMN and neuromelanosis (including nonmelanocytic CNS lesions) are caused by somatic mosaicism for *NRAS* codon 61 mutations in a progenitor cell within the neuroectoderm in patients with this condition. Loss of heterozygosity was associated with the timing of progression to malignancy in two cases, suggesting a central role for the mosaic mutation in a multistep model of melanoma in this condition.

MATERIALS AND METHODS

Subjects

This study was approved by the Great Ormond Street Hospital for Children and the Institute of Child Health Research Ethics Committee, and complied with the Declaration of Helsinki Principles. Samples were obtained from 57 patients with multiple CMN recruited prospectively from the Paediatric Dermatology outpatient department of the Great Ormond Street Hospital for Children between 2006 and 2012, and from 2 patients recruited retrospectively, who had neurological or malignant samples stored in the Pathology department. All patients recruited prospectively had a blood sample taken for DNA extraction. Where prospectively recruited patients underwent routine cutaneous or neurological surgery for clinical reasons during the study period, tissue samples were obtained for DNA extraction subject to an extra level of written consent. A subset of these patients also consented to a punch biopsy of unaffected skin being taken for this research. This method of collecting tissue in only those who were having surgery for cutaneous or neurological reasons was chosen to be the least invasive for participants, and had the effect of increasing the collection of neurological and malignant samples. This was desirable in the investigation of mosaicism within the CNS.

A single experienced assessor (VAK) performed clinical phenotyping (Table 2). The severity of cutaneous lesions was classified by the total number of lesions, and the projected adult size of the largest lesion, which is the best-available and the most widely used classification of CMN (Ruiz-Maldonado, 2004; Krengel *et al.*, 2011). Magnetic resonance imaging scans of the CNS were performed where clinically indicated (as per published protocols; Kinsler *et al.*, 2008).

Selective amplification of mutant alleles

DNA was extracted from fresh tissue using the DNeasy Blood and Tissue Kit (Qiagen) and from paraffin-embedded tissue using Ambion RecoverAll total nucleic acid extraction kit for FFPE (Life Technologies). Site-directed mutagenesis of two forward primers was used to

Table 2. Clinical phenotype of 15 patients from whom tissue was obtained

Case number	Age at the time of study (years)	PAS	Total number of nevi at enrollment	Routine CNS MRI findings in the first year of life	Additional subsequent CNS MRI progression	Clinical CNS findings	Melanoma	At least three characteristic facial features
1	8.29	40–60 cm	>200	Parenchymal neuromelanosis, two congenital spinal neurocristic hamartomata, Dandy–Walker malformation	Hydrocephalus, spinal syrinx, diffuse leptomeningeal melanocytosis stable for 7 y	Wheelchair bound, developmental delay, loss of sensation in one arm, seizures	No	Yes
2	16.28	40–60 cm	20–50	Normal	Not repeated	Speech delay, seizures at puberty, all resolved	No	No
3	12.48	20–40 cm	100–200	Normal	Not repeated	None	No	Yes
4	2.45	>60 cm	100–200	Parenchymal neuromelanosis	Not repeated	None	No	Yes
5	Deceased age 7 y	10–20 cm	50–100	Normal	Normal at 7 y	None	Primary in largest CMN, metastatic to lymph nodes	Yes
6	Deceased age 2 y	>60 cm	50–100	Multiple foci of parenchymal neuromelanosis	Diffuse progressive leptomeningeal melanocytosis, hydrocephalus	Progressive spinal cord compression	Leptomeningeal disease metastatic to abdomen via ventriculoperitoneal shunt	Yes
7	9.12	40–60 cm	>200	Parenchymal neuromelanosis	Not repeated	None	No	No
8	2.94	No single larger lesion	10–20	Parenchymal neuromelanosis nonmelanocytic dural deposits	No change on annual scans	None	No	Yes
9	18.07	20–40 cm	>200	Parenchymal neuromelanosis	No change over time	Seizures, mild developmental delay	No	Yes
10	2.04	10–20 cm	<10	Normal	Not repeated	None	No	Yes
11	4.24	10–20 cm	2	Frontal lobe meningioma	Postsurgical changes only	None pre- or post-resection	No	Yes
12	Deceased age 10 y	>60 cm	100–200	Not done	Cerebellar melanoma, diffuse leptomeningeal melanocytosis	None before melanoma, raised intracranial pressure and progressive spinal cord compression	Primary in cerebellum, diffuse progressive leptomeningeal melanocytosis, metastatic to liver	Yes
13	22.98	10–20 cm	2	Not done	Hydrocephalus, choroid plexus papilloma	Of raised intracranial pressure pre-resection, none post-resection	No	Not done
14	17.06	>60 cm	>200	Normal	Not repeated	None	No	Yes
15	2.79	No single larger lesion	100–200	Parenchymal neuromelanosis	Not repeated	None	No	Yes

Abbreviations: CMN, congenital melanocytic nevus; CNS, central nervous system; MRI, magnetic resonance imaging; PAS, projected adult size of largest CMN; y, year.
Total number of nevi includes the largest CMN.

introduce a single-nucleotide change at chr1:115256535 C>G or chr1:115256532 G>T, creating recognition sites GTNNAC and TGTACA only in the wild-type sequence, for restriction enzymes *Hpy*166II and *Bsr*G1, respectively (New England Biolabs, Ipswich, MA). Standard PCR (94 °C for 2 minutes, 35 cycles of 94 °C for 30 seconds/54 °C for 1 minute/72 °C for 1 minute, 72 °C for 2 minutes) was followed by enzymatic digestion and a subsequent hemi-nested amplification (94 °C for 2 minutes, 25 cycles of 94 °C for 30 seconds/60 °C for 30 seconds/72 °C for 30 seconds, 72 °C for 2 minutes), with Sanger sequencing with the reverse primer after each PCR. Where wild-type results were obtained, two further cycles of digestion and nested PCR were done, as used in the detection of mutant *GNAS*

alleles in mosaic fibrous dysplasia (Candeliere *et al.*, 1997). *NRAS* primer sequences are shown in Supplementary Table S1 online. Enzymatic digestion used 5 µl PCR product, 1 µl enzyme (1,000 U ml^{-1}), and 45 µl New England Biolabs Buffer 4, incubated at 37 °C for 15 minutes. Then, 2 µl of this solution was used in the ensuing 20 µl PCR reaction. Enzymatic digestion of the wild-type allele significantly increased *NRAS* mutation detection (Supplementary Figure S1online); however, clear detection of the mutant allele was possible in all cases after the first cycle of digestion, with no mutations discovered with subsequent cycles. All sequencing was performed on an ABI3130XL Sequencer (Applied Biosystems). Sequencing data were analyzed using Sequencher (Gene Codes, Ann Arbor, MI) without access to the sample identifiers.

Quantification of mosaicism

Quantification of mosaicism was performed using Mutation Surveyor software (Soft Genetics, State College, PA) to calculate the simplified allele ratio. Using this method the software looks at the relative fluorescent units of the normal peak and the mutant peak, and uses the following formula to work out the mutant percentage:

100 × (Mutant peak intensity/(Mutant peak intensity + Normal peak intensity)).

To verify the accuracy of this method of quantification, TA cloning of heterozygous Q61K samples was performed using TOPO TA Cloning Kit (Life Technologies), and homozygous and wild-type alleles isolated and confirmed by sequencing. Homozygous and wild-type alleles were mixed in known quantities to produce samples of 100, 50, 25, 10, 5, and 1% mosaicism for Q61K. These were sequenced in triplicate and the percentage mosaicism measured blind using the method described above. The correlation between the measured and known percentages of mosaicism on linear regression was high ($r^2 = 0.990$, $P < 0.001$) (Supplementary Figure S1 online).

Array CGH

Array CGH was performed on five samples: two melanoma samples and two proliferative nodule samples from patients 5 and 12, and one of nonmalignant clinically stable diffuse leptomeningeal melanocytosis sample from patient 1 (patient details are in Table 2). Then, 1–3 µg of patient and pooled-control DNA was labeled with fluorescent dyes Cy3 and Cy5, respectively, and hybridized to NimbleGen (Madison, WI) 135K oligonucleotide arrays. Data were analyzed using InfoQuant (London, UK) CGHFusion (version 5.7.0). The melanoma samples were also sequenced for *BRAF* V600E mutations using primers forward and reverse 5′-GCTCGCCCAGGAGTGC-CAAG-3′/ 5′-TGGCCCTGAGATGCTGCTGA-3′.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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