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

Veronica A. Kinsler1,2, Anna C. Thomas2, Miho Ishida2, Neil W. Bulstrode3, Sam Loughlin4, Sandra Him3, Jane Chalker3, Kathryn McKenzie6, Sayeda Abu-Amero5, Olga Slater7, Estelle Chanudet8, Rodger Palmer3, Deborah Morrogh4, Philip Stanier9, Eugene Healy10, Neil J. Sebire11,12 and Gudrun E. Moore2

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 neurocutaneous 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>T, 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 neuroepithelial lesions in the majority of cases.

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 (Ruíz-Maldonado et al., 1997), and this disconnect is likely to be due to developmental intraparenchymal lesions below the resolution of

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Postzygotic NRAS and polymorphisms in melanocortin 1 receptor reported a role for germline and somatic RAS mutations 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
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 neurocrystic 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

| Case number | Unaffected skin | CMN | Unaffected meninges | Diffuse leptomeningeal melanocytosis | Primary melanoma | Metastatic melanoma | CNS tumor (non-melanocytic) | Blood |
|-------------|----------------|-----|---------------------|------------------------------------|----------------|-------------------|--------------------------|-------|
| 1           | ■■             |■■■■ |                     |                                    |                |                   |                          | ■     |
| 2           | ■              |■■   |                     |                                    |                |                   |                          | ■     |
| 3           | ■              |■■   |                     |                                    |                |                   |                          | ■     |
| 4           | ■              |■■   |                     |                                    | ■              |                   |                          | ■     |
| 5           | ■              |■■■■ |                     |                                    |                |                   |                          | ■     |
| 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.
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-NRASQ61K fusion protein into developing zebrafish leads to multiple cutaneous nevi, and transgenic zebrafish overexpressing NRASQ61K in developing melanocytes showed widespread hyperpigmentation (Dovey et al., 2009). A transgenic NRASQ61K 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
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 Hpy166II and BsrG1, respectively (New England Biolabs, Ipswich, MA). Standard PCR (94°C for 2 minutes, 35 cycles of 94°C for 30 seconds/60°C for 30 seconds/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.

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 nevromelanosis, two congenital spinal neurocrystall nevroma, 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 nevromelanosis | 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 nevromelanosis | 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 nevromelanosis | Not repeated | None | No | No |
| 8           | 2.94                             | No single larger lesion | 10–20 | Parenchymal nevromelanosis nonmelanocytic dural deposits | No change on annual scans | None | None | Yes |
| 9           | 18.07                            | 20–40 cm | >200 | Parenchymal nevromelanosis | 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 nevromelanosis | 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.
alleles in mosaic fibrous dysplasia (Candelieri 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 S1 online); 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:

\[ 100 \times (\text{Mutant peak intensity} / (\text{Mutant peak intensity} + \text{Normal peak intensity})) \]

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 two nodular naevi of the normal peak and the mutant peak, and uses 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:

\[ 100 \times (\text{Mutant peak intensity} / (\text{Mutant peak intensity} + \text{Normal peak intensity})) \]

Conflicts 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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