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ORIGINAL ARTICLE

NRAS Mutation Is the Sole Recurrent Somatic Mutation in Large Congenital Melanocytic Nevi

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Congenital melanocytic nevus (CMN) is a particular melanocytic *in utero* proliferation characterized by an increased risk of melanoma transformation during infancy or adulthood. *NRAS* and *BRAF* mutations have consistently been reported in CMN samples, but until recently results have been contradictory. We therefore studied a series of large and giant CMNs and compared them with small and medium CMNs using Sanger sequencing, pyrosequencing, high-resolution melting analysis, and mutation enrichment by an enhanced version of *ice*-COLD-PCR. Large–giant CMNs displayed *NRAS* mutations in 94.7% of cases (18/19). At that point, the role of additional mutations in CMN pathogenesis had to be investigated. We therefore performed exome sequencing on five specimens of large–giant nevi. The results showed that *NRAS* mutation was the sole recurrent somatic event found in such melanocytic proliferations. The genetic profile of small–medium CMNs was significantly different, with 70% of cases bearing *NRAS* mutations and 30% showing *BRAF* mutations. These findings strongly suggest that *NRAS* mutations are sufficient to drive melanocytic benign proliferations *in utero*.

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INTRODUCTION

Congenital melanocytic nevus (CMN) is a benign clonal melanocytic proliferation that develops *in utero*. CMN is classified by its projected adult size (PAS), may be associated with neurocutaneous melanosis (DeDavid *et al.*, 1996), and moreover exhibits a risk of melanoma transformation. Indeed, prospective studies have reported a rate of 4–5% of melanomas in patients with CMNs >20 cm PAS after 5 years (Ruiz-Maldonado *et al.*, 1992; Marghoob *et al.*, 1996). The risk of melanomas appears proportional to the lesion size, reaching up to a 10–15% risk for lesions >40 cm in diameter (Krengel *et al.*, 2006). Neurological abnormalities, numerous satellite nevi, and a truncal location are also reported in patients with CMNs >40 cm PAS (Hale *et al.*, 2005; Bett, 2006). On the other hand, familial cases of CMNs have only rarely been reported (Frieden and Williams, 1994; de Wijn *et al.*, 2010). CMN does not follow a Mendelian pattern and is rather considered to be the consequence of a causative somatic mutation. CMNs have consistently been reported to harbor *NRAS* and *BRAF* mutations (Papp *et al.*, 1999, 2005; Ichii-Nakato *et al.*, 2006; Bauer *et al.*, 2007; Dessars *et al.*, 2009; Qi *et al.*, 2011; Wu *et al.*, 2011). Several studies have described *NRAS* mutations in CMNs (Bauer *et al.*, 2007; Dessars *et al.*, 2009; Wu *et al.*, 2011; Kinsler *et al.*, 2013), whereas others detected the presence of *BRAF* mutations (Papp *et al.*, 1999, 2005; Wu *et al.*, 2007; Qi *et al.*, 2011). In view of these discrepancies, we aimed to study *NRAS* and *BRAF* mutations in our series of CMNs using different powerful sequencing techniques. Moreover, in melanomagenesis, *NRAS* mutations are known to lead to transformation by cooperating with other genetic events. Similarly, additional mutations may intervene in CMN pathogenesis. In order to answer the question of other pathways involved in CMN, we performed unbiased next-generation whole-exome sequencing using large CMNs and matched control blood specimens.

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Abbreviations: CMN, congenital melanocytic nevus; HRM, high-resolution melting; PAS, projected adult size; SNV, single-nucleotide variation; WT, wild type

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RESULTS

NRAS mutations in large and giant CMNs analyzed by four different sequencing techniques

A total of 19 patients diagnosed with large or giant CMNs were included in the study. The clinical characteristics are reported in Table 1. The median age of the patients was 8 months (range, 2–60 months). The male/female sex ratio was 0.73 (8 males/11 females). All body localizations were represented: 7 of 19 nevi (36.9%) involved the head, 8 of 19 nevi (42.1%) involved the arm and/or trunk, and 4 of 19 (21%) the legs. Neurological symptoms were found in none of the 19 patients; ultrasonographic evaluations and magnetic resonance images were normal in all patients who underwent such examinations. No history of familial CMN was recorded in any patient.

DNA samples were processed using three standard techniques. *NRAS* and *BRAF* mutations were screened using Sanger sequencing. Mutations in *NRAS* exon 3 were also assessed using pyrosequencing and high-resolution melting (HRM) analysis in order to further improve the sensitivity of mutation detection. Using these three techniques, only one large CMN harbored a *BRAF* mutation. We showed that 16 large or giant CMNs bore an *NRAS* mutation, with 8 c.181C>A, p.Q61K mutations and 8 c.182A>G, p.Q61R mutations. Two nevi (patients 7 and 12) displayed neither *BRAF* nor *NRAS* mutations (Table 2).

A minor *NRAS* clone could still remain undetectable at this stage. To definitely solve this question, we harvested additional specimens (from 4 other areas for patient 12) as well as performed enhanced version of *ice*-COLD-PCR (E-*ice*-COLD-PCR; for patients 7 and 12). This technique relies on a chemically modified, nonextendable oligonucleotide complementary to the wild-type (WT) allele sequences that blocks amplification of WT alleles and thereby ''enrich'' mutated alleles. These mutated alleles were subsequently analyzed by pyrosequencing. Interestingly, a clonal *NRAS* Q61R mutation was found by E-*ice*-COLD-PCR in both of these large CMNs (patients 7 and 12). Therefore, this high-sensitivity sequencing technique detected *NRAS* mutations in two large CMNs (10.5%) previously classified as *BRAF* and *NRAS* WT using standard techniques. Overall, an *NRAS* mutation was detected in 94.7% of large or giant CMNs (18/19); 8 out of 19 nevi (42.1%) bore the c.181C>A, p.Q61K mutation whereas 10 out of 19 nevi (52.6%) displayed the c.182A>G, p.Q61R mutation. Finally, one large CMN (patient 9) harbored a *BRAF* mutation without any *NRAS* mutation. Of note, the first sample of patient 12 nevus did not display a *BRAF* or *NRAS* mutation even using E-*ice*-COLD-PCR. However, when four additional nevus areas (specimens) were selected and screened, two out of four revealed the presence of a c.182A>G, p.Q61R *NRAS* mutated clone.

Table 1. Clinical characteristics of the 19 large and giant CMNs

Patient	Age	Sex	Tissue site	PAS (cm)	Ruiz-Maldonado classification[1]	NCM/neurological symptoms	Familial CMN	Histological features
1	3.5 Years	Female	Head	>20–30	Giant	No	No	Compound
2	5 Years	Female	Arm	>20–30	Giant	No	No	Compound
3	2 Months	Female	Leg	>10–20	Large	No	No	Compound
4	19 Months	Male	Arm	>10–20	Large	No	No	Compound
5	21 Months	Female	Leg	>10–20	Large	No	No	Compound
6	5 Years	Male	Head	>10–20	Large	No	ND	Compound
7	3 Months	Male	Trunk	>10–20	Large	ND	ND	Compound
8	7 Months	Male	Trunk	>40–60	Giant	No	ND	Compound
9	25 Months	Male	Leg	>10–20	Large	No	No	Compound
10	4 Months	Male	Head	>10–20	Large	No	No	Compound
11	4 Months	Female	Trunk	>20–30	Giant	No	ND	Compound
12	5 Months	Female	Head	>10–20	Large	No	No	Compound
13	9 Months	Female	Head	>20–30	Giant	No	No	Dermal
14	13 Months	Male	Head	>10–20	Large	No	No	Compound
15	3 Months	Male	Head	>20–30	Giant	ND	ND	Compound
16	8 Months	Female	Arm	>10–20	Large	No	ND	Compound
17	6 Months	Female	Arm	>20–30	Giant	No	No	Compound
18	4 Months	Female	Trunk	>10–20	Large	No	No	Dermal
19	9 Months	Female	Leg	>10–20	Large	No	No	Compound

Abbreviations: CMN, congenital melanocytic nevus; NCM, neurocutaneous melanosis; ND, nondetermined; PAS, projected adult size.
[1]Classification by PAS as defined in Ruiz-Maldonado (2004).

Table 2. Genetic characteristics of the 19 large and giant CMNs

Patient	Sanger	NRAS exon 3		
		Pyrosequencing	HRM[1]	E-*ice*-COLD-PCR
1	WT	WT	NRAS3 mutated	NRAS3 Q61K
2	NRAS3 Q61R	NRAS3 mutated	NRAS3 mutated	
3	NRAS3 Q61K	NRAS3 mutated	NRAS3 mutated	
4	NRAS3 Q61R	NRAS3 mutated	NRAS3 mutated	
5	NRAS3 Q61K	NRAS3 mutated	NRAS3 mutated	
6	NRAS3 Q61K	NRAS3 mutated	NRAS3 mutated	
7	ND	WT	WT	NRAS3 Q61R
8	NRAS3 Q61R	NRAS3 mutated	NRAS3 mutated	
9	BRAF V600E	WT	WT	
10	NRAS3 Q61R	NRAS3 mutated	NRAS3 mutated	
11	NRAS3 Q61K	NRAS3 mutated	NRAS3 mutated	
12	WT	WT	WT	NRAS3 Q61R
13	NRAS3 Q61R	NRAS3 mutated	NRAS3 mutated	
14	NRAS3 Q61R	NRAS3 mutated	NRAS3 mutated	
15	NRAS3 Q61K	NRAS3 mutated	NRAS3 mutated	
16	NRAS3 Q61K	NRAS3 mutated	NRAS3 mutated	
17	NRAS3 Q61R	NRAS3 mutated	NRAS3 mutated	
18	NRAS3 Q61R	NRAS3 mutated	NRAS3 mutated	
19	NRAS3 Q61K	NRAS3 mutated	NRAS3 mutated	

Abbreviations: CMN, congenital melanocytic nevus; E-*ice*-COLD-PCR, enhanced version of *ice*-COLD-PCR; HRM, high-resolution melting analysis; ND, nondetermined; WT, wild type.

[1]High-resolution melting analysis. Sanger sequencing was used to screen *NRAS* exon 2 and 3 and *BRAF* exon 15 mutations. Pyrosequencing and high-resolution melting analysis were used to screen *NRAS* exon 2 and 3 mutations. Enhanced-*ice*-COLD-PCR was used to screen *NRAS* exon 3 mutation.

Table 3. Results of whole-exome sequencing of five CMN samples and matched blood DNA

Gene	Chr		cDNA shift	Peptide shift	SIFT prediction	Patient 6	Patient 12	Patient 14	Patient 15	Patient 16
NRAS	1	SNP	c.181C>A	p.Q61K	Damaging	1	0	0	1	1
NRAS	1	SNP	c.182A>G	p.Q61R	Damaging	0	0	1	0	0

Abbreviations: cDNA, complementary DNA; CMN, congenital melanocytic nevus; SNP, single-nucleotide polymorphism.

***NRAS* mutation as the sole recurrent somatic mutation confirmed by whole-exome sequencing of large and giant CMNs**

We asked therefore whether additional mutations beside *NRAS* were needed to drive CMN pathogenesis. To answer this question, we selected DNA from five patients with large or giant CMNs with matched nontumor peripheral blood DNA available. Whole-exome sequencing was performed on these five sample pairs in order to detect somatic mutations in the nevi (Table 3). Median coverage of the exome sequencing was 54× (range, 51–60). Analysis revealed a total of nine somatic nonsynonymous mutations involving eight genes. As expected, 4 of the 5 tumors harbored the previously confirmed *NRAS* mutation (patients 6, 14, 15, and 16). Visual inspection of the reads did not identify any other mutations in the vicinity of the *NRAS* Q61 mutation. We also evaluated the percentage of mutated cells within a nevus harboring the *NRAS* mutation. It ranged from 10 to 40% in these 4 patients analyzed using whole-exome sequencing: 25% in patient 6 (13 mutated/51 nonmutated tags), 10% in patient 14 (8 mutated/86 nonmutated tags), 40% in patient 15 (22 mutated/55 nonmutated tags), and 19% in patient 16 (14 mutated/74 nonmutated tags). Interestingly, patient 12 nevus, which revealed a NRAS mutated subclone only after an extensive screening using E-*ice*-COLD-PCR sequencing of several areas, displayed 1 mutated tag of *NRAS* out of 79 tags in the nevus versus no identified mutated tag in matched blood DNA (number of total tags detected 64). Therefore, both E-*ice*-COLD-PCR and exome sequencing techniques led to corresponding results. Besides *NRAS*, and using our criteria (3 mutated tags in nevus, and a total of 10 tags covered in nevus and normal at minimum), exome sequencing revealed the presence of seven other non-*NRAS*, non-*BRAF*, nonsynonymous mutations

Table 4. Clinical and genetic characteristics of the 20 small and medium CMNs

Sample	Age	Sex	Tissue site	PAS (cm)	Histological features	Sequencing[1]	E-*ice*-COLD-PCR
20	35 Months	Female	Arm	1.5–10	Compound	WT	NRAS3 Q61K
21	4 Months	Female	Head	1.5–10	Compound	NRAS3 Q61R	
22	10 Months	Male	Arm	1.5–10	Compound	NRAS3 Q61R	
23	16 Years	Female	Leg	1.5–10	Dermal	NRAS3 Q61K	
24	11 Months	Male	Head	1.5–10	Compound	WT	NRAS Q61R
25	9 Months	Female	Trunk	1.5–10	Compound	BRAF V600E	NRAS3 WT
26	16 Months	Female	Head	1.5–10	Compound	WT	NRAS3 Q61R
27	44 Months	Male	Trunk	1.5–10	Compound	ND	NRAS3 Q61K
28	12 Years	Male	Head	1.5–10	Compound	BRAF V600E	NRAS3 WT
29	38 Months	Female	Arm	1.5–10	Compound	WT	NRAS3 Q61K
30	8.5 Years	Female	Trunk	1.5–10	Compound	BRAF V600E	
31	6 Years	Female	Arm	1.5–10	Compound	NRAS3 Q61K	NRAS3 Q61K
32	16 Months	Male	Leg	1.5–10	Compound	BRAF V600E	
33	5 Years	Female	Leg	1.5–10	Compound	WT	NRAS3 Q61R
34	10 Years	Male	Head	<1.5	Compound	BRAF V600E	
35	4 Months	Female	Arm	1.5–10	Compound	BRAF V600E	
36	22 Months	Female	Trunk	1.5–10	Compound	WT	NRAS3 Q61K
37	6 Years	Female	Leg	1.5–10	Compound	ND	NRAS3 Q61K
38	33 Months	Male	Leg	1.5–10	Compound	WT	NRAS3 Q61R
39	7 Months	Male	Trunk	1.5–10	Compound	NRAS3 Q61K	NRAS3 Q61K

Abbreviations: CMN, congenital melanocytic nevus; E-*ice*-COLD-PCR, enhanced version of *ice*-COLD-PCR; ND, nondetermined; PAS, projected adult size; WT, wild type.
Mutation enrichment by enhanced-*ice*-COLD-PCR was used to screen *NRAS* exon 3 mutations.
[1]Sanger sequencing was used to screen *NRAS* exon 2 and 3 and *BRAF* exon 15 mutations followed by pyrosequencing and high-resolution melting analysis in order to screen *NRAS* exon 2 and 3 mutations.

involving *BAGE5*, *C5orf42*, *EPS15*, *ETV2*, *KRT77*, *NKIRAS2*, and *TRPV4*. At this stage, we had to confirm whether these results corresponded to real mutations or to nonreproducible artifacts. Using two different sequencing techniques, i.e., Sanger sequencing (Supplementary Table S1 online) and a sensitive allele-specific PCR approach, we failed to confirm the presence of these mutations, suggesting that they corresponded to false positives of the technique. Therefore, *NRAS* mutation appears to be the sole recurrent somatic mutation identified in large and giant CMNs.

Mixed pattern of *NRAS* and *BRAF* mutations in small and medium CMNs

A second group of smaller CMNs was compared with the previous group of CMNs: 20 patients diagnosed with small or medium CMNs were also studied. The median age of the patients was 50 months, whereas the mean age was 35 months. The male/female sex ratio was 0.67 (8 males/12 females). All body localizations were represented: 5 of 20 nevi (25%) involved the head, 10 of 20 nevi (50%) involved the arm and/or trunk, and 5 of 20 (25%) the leg. Therefore, in terms of sex ratio and body localization of nevi, both groups were comparable.

Small and medium CMNs were analyzed for *NRAS* and *BRAF* mutations using the various techniques previously described: Sanger sequencing, pyrosequencing, HRM analysis, and mutation enrichment by E-*ice*-COLD-PCR (Table 4).

An *NRAS* mutation was detected in 14 out of 20 (70%) small–medium CMN: 8 nevi (40%) bore the c.181C>A, p.Q61K mutation and 6 nevi (30%) bore the c.182A>G, p.Q61R mutation. A total of 30% (6/20) of small–medium CMNs harbored a *BRAF* mutation. Therefore, the mutational profile of these nevi was different from the large CMN mutational profile, with a lower percentage of *NRAS* mutations and a higher percentage of *BRAF* mutations. Of note, only 5 *NRAS* mutated nevi (25%) were detected using the three conventional techniques. The high-sensitivity sequencing technology showed low-frequency mutated clones in small–medium lesions as it revealed *NRAS* mutations in 9 nevi (45%) previously classified as *NRAS* WT using standard technologies, whereas this was the case in only 2 large CMNs (10.5%).

DISCUSSION

NRAS is one of the three major isoforms of the RAS family of GTPases proteins. Through the downstream activation of four major signaling pathways mitogen-activated protein kinase,

phosphatidylinositide 3 kinase, phospholipase C/protein kinase C, and RalGDS, NRAS is involved in cell growth. All reported *NRAS* gene mutations occur in exon 2 or exon 3, with 65% of mutations occurring in exon 3 at codon 61. The most frequent amino acid substitutions are Q61K and Q61R (Takata and Saida, 2006). These substitutions result in a constitutively active NRAS protein, unable to cleave guanosine triphosphate.

Using an unbiased series of patients, we found the presence of *NRAS* mutations in 94.7% and 70% of large–giant and small–medium CMN cases, respectively. *NRAS* mutations have been detected at low percentage in malignant melanoma as well as in acquired melanocytic nevi (Poynter *et al.*, 2006). However, *NRAS* mutations have been closely associated with CMNs. Previous studies detected variable levels of *NRAS* mutation in CMNs. These vary between 22 and 80% in affected cutaneous and neurological tissues of patients (Carr and Mackie, 1994; Ichii-Nakato *et al.*, 2006; Dessars *et al.*, 2009; Wu *et al.*, 2011; Kinsler *et al.*, 2013). In view of these differences, our study confirms that CMNs display a high incidence of *NRAS* mutations, highlighting the role of this pathway in the disease.

Malignant tumors are usually featured by several mutations that accumulate during neoplastic proliferation (Hanahan and Weinberg, 2011). It was therefore mandatory to assess the eventual intervention of other mutated pathways. We therefore selected 5 patients out of our series, including patient 12 initially negative for *BRAF* and *NRAS* mutations when screened by four sequencing techniques. The nevus specimens were sequenced using next-generation whole-exome analysis and compared with the patient's peripheral blood concomitantly analyzed. This technique is currently the most powerful one to assess the presence of any somatic mutation in an acquired disease (Shyr and Liu, 2013). Surprisingly, the unique recurrent mutation detected was *NRAS*. Patient 12, who was found positive for an *NRAS* mutated subclone only after screening of 4 additional nevus areas using E-*ice*-COLD-PCR, also displayed an *NRAS* clone (1 mutated tag in 79) in whole-exome sequencing, consistent with the fact that a minor *NRAS* mutated clone existed in this case.

Our study focuses on exonic regions, and further studies are needed to assess that intronic mutations are not involved. Indeed, exome sequencing only allows the identification of somatic mutations present in coding areas. Our study does not provide information concerning intronic regions, nor was it conducted in a way to analyze the significance of germline mutations. Nevertheless, Kinsler *et al.* (2012) have reported a role for germline *MC1R* genotype in CMNs, dismissing *MC1R* as a somatic mosaic candidate. Exome sequencing cannot depict DNA copy number changes such as microdeletions or duplications. These request performing comparative genomic hybridization array. However, previous studies from Bastian (2003) and Bastian *et al.* (1999) have indicated the absence of such abnormalities in congenital nevi and acquired nevi, except in Spitz nevi. Altogether, our data combined with these other results indicate that no other somatic event than the *NRAS* mutation at codon 61 is responsible for the development of congenital nevi to the exclusion of possible intronic mutations. Of note, other examples showed that tumors including malignant ones may rely on a unique mutation. Indeed, in rhabdoid child tumors, exome sequencing analysis indicated that biallelic loss of a subunit of the SWI/SNF chromatin remodeling complex was solely responsible for this malignancy (Lee *et al.*, 2012). Moreover, the recent demonstration that identical somatic codon 61 *NRAS* mutations were found in different CMN lesions, melanomas as well as in neurological lesions from the same patients, also supports the causative role of the *NRAS* mutation in CMN genesis (Kinsler *et al.*, 2013). In the same study, Kinsler *et al.* (2013) report that the *NRAS* mutation found in affected patients segregated only in neural crest–derived cells or tumors such as choroid plexus papilloma, neurocristic hamartoma, meningioma, or diffuse leptomeningeal melanocytosis as the same *NRAS* mutation was found in these separate anatomical sites. Conversely, *NRAS* mutation was absent from other tissues such as the blood of patients. This supports the hypothesis of a single-mutated precursor cell giving rise to the nevus as well as rare neural crest–derived tumors.

We report *BRAF* mutations in 5.2% (1/19) and 30% of large–giant and small–medium CMNs, respectively. Previous studies found conflicting data indicating the presence of a *BRAF* mutation between 0% (Bauer *et al.*, 2007; Wu *et al.*, 2011) and 86% in nevi documented either at birth by parents testimony (Ichii-Nakato *et al.*, 2006), medical records (Wu *et al.*, 2007; Qi *et al.*, 2011), or selected by histological criteria (Pollock *et al.*, 2003). Such histological selection is controversial as it is known that acquired melanocytic nevi may mimic histologically a congenital pattern. Using our sensitive techniques with nevi assessed both by parents' testimony and medical records, we were able to find that the frequency of *BRAF* mutation does not exceed 30%, showing that it was less associated with CMN than *NRAS* mutation.

In our study, patients have been categorized using Ruiz-Maldonado classification (Ruiz-Maldonado, 2004). The risk of melanoma appears nearly absent in small and medium nevi that have been grouped here. In contrast, malignant transformation develops in up to 5% in series of nevi with PAS >20 cm (Ruiz-Maldonado *et al.*, 1992; Marghoob *et al.*, 1996). Importantly, in the literature, a precise estimation of such risk is not known for the large nevi (>10–20 cm) that we have therefore grouped with the giant nevi (>20 cm). Interestingly, the rate of *NRAS* mutation appears similar in these large (11/12, 92%) and giant (7/7, 100%) specimens.

CMNs may have different genetic signatures, with either *NRAS* or *BRAF* mutations, *BRAF* mutations having a lower incidence rate in CMN >20 cm PAS, and a higher incidence rate in small CMNs. On the other hand, $\sim$79% (376 of 479) of acquired nevi harbor *BRAF* mutations (Pollock *et al.*, 2003; Ichii-Nakato *et al.*, 2006; Ross *et al.*, 2011). Of note, these acquired nevi do not transform more frequently than melanocytes interspersed in normal epidermis outside a nevus. Therefore, the risk of malignant transformation does not parallel the expression of *BRAF* mutation. Interestingly, as compared with *BRAF* mutated melanomas or *NRAS*/*BRAF* WT melanomas, melanomas with *NRAS* mutations are more likely to be thicker tumors and to have a higher mitotic rate

(Devitt *et al.*, 2011; Ascierto *et al.*, 2013). One may therefore consider that *BRAF* mutations may not be as important as *NRAS* mutations in the risk of melanoma arising from benign nevi in general and CMNs in particular. Nevertheless, in order to fully answer that question, studies comparing melanoma incidence rate in *NRAS* mutated CMNs and in *BRAF* mutated CMNs would be needed.

In conclusion, our study strongly suggests that codon 61 *NRAS* mutations in melanocytes are sufficient to drive *in utero* proliferation. This result should encourage to examine alternative medical treatments to surgery.

MATERIALS AND METHODS

Study approval

The study was approved by the institutional independent ethics committee (Comité de Protection des Personnes Ile-de-France V) and complied with the Declaration of Helsinki Principles. The patients' guardians provided written informed consent before their participation.

Subjects

Clinical phenotyping was performed by AP, NK, and SG. Classification was done using the PAS of the largest lesion (Ruiz-Maldonado, 2004). A total of 19 patients with large or giant CMNs and 20 patients with small or medium CMNs were included prospectively in the study and nevus samples collected. Tumor tissues and matched blood from five patients diagnosed with large or giant CMNs were collected for the whole-exome sequencing analysis.

DNA extraction and genotyping

DNA was extracted using standard techniques: from fresh tissue using the DNeasy Blood and Tissue Kit (Qiagen, Courtaboeuf, France) and from paraffin-embedded tissue using QIAamp DNA FFPE Tissue Kit (Qiagen, France). The DNA was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Several genotyping methods were used to identify BRAF exon 15 and NRAS exons 2 and 3 mutations using specific primer sets. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were used for control of DNA quality. Positive, negative, and no-DNA controls were included in each run.

Sanger sequencing

A sequence of ~500 nucleotides surrounding the mutation site was amplified using the Finnzyme Phusion High-Fidelity DNA polymerase (Thermo Scientific, Villebon-sur-Yvette, France). Depending on the CG content of the amplified sequence, DMSO was added to the reaction. PCR products were then purified on 96-well Multiscreen-PCR filtration plates (Millipore, Molsheim, France). The following NRAS and BRAF primers were used: BRAF_F, 5′-TGCTCTGATAGGAAAATGAGATCTAC-3′ and BRAF_R, 5′-GGCCAAAAATTTAATCAGTGG-3′; NRAS2_F, 5′-GAACCAAATGGAAGGTCACA-3′ and NRAS2_R, 5′-TGGGTAAAGATGATCCGACA-3′; NRAS3_F, 5′-CCAGATAGGCAGAAATGGGCT-3′ and NRAS3_R, 5′-GCTCTATCTTCCCTAGTGTGGT-3′. A listing of the other primers used, annealing temperatures, and expected length of PCR products is given in Supplementary Table S1 online. Sanger sequencing analyses were conducted according to the manufacturer's protocol using respectively the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Courtaboeuf, France), on an ABI3130 running ABI Prism DNA Sequence Analysis Software (Applied Biosystems).

Pyrosequencing

Pyrosequencing was performed according to standard procedures using PyroMark Gold Q96 (Qiagen, Hilden, Germany) reagents and a PyroMarkTM Q96 ID instrument. Pyrograms outputs were analyzed with PyroMarkTM Q96 ID Software (Qiagen, Germany) using the allele quantification mode.

High-resolution melting

HRM analyses were conducted according to the manufacturer's protocol using the LC480 HRM Scanning Master (Roche, Boulogne-Billancourt, France) on a LightCycler 480 (Roche).

Enrichment of NRAS mutations by E-ice-COLD-PCR

For codon 61 *NRAS* mutation amplification, an E-*ice*-COLD-PCR assay was designed following the standard protocol (How Kit *et al.*, 2013). PCR primers (NRAS_F 5′-GTTGGACATACTGGATAC-3′ and NRAS_R Biotin-5′-ATGACTTGCTATTATTGATG-3′) were designed with Beacon Designer 8 (Premier Biosoft, Palo Alto, CA) and purchased from Biotez (Berlin, Germany). Primers amplified a 113-bp PCR product flanking the codons of interest. The nonextendable blocker probe of 50-nucleotide length (NRAS_BK_LNA 5′-GATACAGCTGGA+C+A+AGAAGAGTACAGTGCCATGAGAGACCAATACATGAG-3′-Phosphate), purchased from TibMolBiol (Berlin, Germany), overlaps 5 nucleotides with the forward PCR primer and presents a triplet of LNA residues centered on codon 61 in order to improve the ΔTM of homoduplexes compared with heteroduplexes when the mismatches are located on the three nucleotides of interest. The critical temperature of 79 °C was determined as described in How Kit *et al.* (2013) using a NRAS c.182A>G mutated cell line (BLM melanoma cells) and WT DNA (Promega, Lyon, France). E-*ice*-COLD-PCR reaction was performed in a Mastercycler Pro S (Eppendorf, Le Pecq, France) and contained 5–25 ng of genomic DNA, 1× HotStar Taq DNA polymerase Buffer, 1.6 mM of additional $MgCl_2$, 200 μM of dNTPs, 200 nM of each primer, 2 U of HotStar Taq DNA polymerase (Qiagen, France), and 10–50 nM of Blocker probe in a final volume of 25 μl. An initial denaturation step was performed for 10 minutes at 95 °C, followed by 6 cycles of standard PCR (30-second denaturation at 95 °C, 20-second annealing at 55 °C, and 10-second elongation at 72 °C) and 44 cycles of E-*ice*-COLD-PCR (20-second denaturation at 95 °C, 30-second blocker annealing at 70 °C, 20-second at 79 °C (critical temperature), 20-second primer annealing at 55 °C, and 10-second elongation at 72 °C) to enrich the mutations. Mutation detection, identification, and quantification were performed by pyrosequencing. Next, 10 μl of amplified E-*ice*-COLD-PCR products were purified and rendered single-stranded on a PyroMark Q96 Vacuum Workstation (Qiagen, France) as previously described (Tost and Gut, 2007). The sequencing primer (NRAS_Gen_F, 5′-TGGACATACTGGATACAGC-3′) was annealed to the target sequence after incubation at 80 °C for 2 minutes.

Sensitivity of the applied sequencing techniques

Using given amounts of mutated DNA as standards, the mutation detection sensitivity for each of the applied sequencing techniques for the *BRAF* and *NRAS* mutations is as follows: *BRAF* screening by

Sanger, 15%; *NRAS* exon 2 and 3 screening by Sanger, 15%; *NRAS* exon 2 and 3 screening by HRM, 5%; and *NRAS* exon 3 screening by *ice*-COLD-PCR, 0.5–1%.

Whole-exome sequencing

Exome capture was performed using Agilent (Santa Clara, CA) SureSelect Human All Exon 50 Mb according to the manufacturer's instructions. Briefly, 3 μg of DNA from each sample was used to prepare the sequencing library through shearing of the DNA followed by ligation of sequencing adaptors. Whole-exome sequencing was performed, and paired-end sequencing (2×76 bp) was carried out using the Illumina (San Diego, CA) HiSeq 2000; the resulting data were analyzed with the Illumina pipeline to generate raw fastq files. The coverage of our samples varied between $51\times$ and $54\times$.

Somatic mutation detection from whole-exome sequencing

After raw paired-end reads from whole-exome resequencing were aligned/mapped to the human genome reference (hg19) and PCR duplicate reads were removed by Mosaik aligner, we then analyzed the resulting alignments using the Bayesian model-based software GigaBayes/FreeBayes (National Institutes of Health, Bethesda, MD) that enables the efficient analysis of billions of aligned short-read sequences (Marth *et al.*, 1999). The program evaluates each aligned base and base quality value at each position to indicate putative single-nucleotide variations (SNVs) and short insertions/deletions (indels), and their corresponding SNV probability value. Base quality values are converted to base probabilities corresponding to each of the four possible nucleotides. Using a Bayesian formulation, a SNV probability value (or indel probability value, as appropriate) is calculated as the likelihood that multiple different alleles are present between the reference genome sequence and the reads aligned at that position. If the probability value exceeds a prespecified threshold, the SNV or indel candidate is reported in the output. In this study, we used a SNV probability cutoff value (0.9) to define a high-confidence SNV or short indel candidate. We also filtered out all known SNVs/indels in the University of California, Santa Cruz (UCSC) dbSNP 135 and 1000 human genome project SNP database, and kept any mutations that are in the Catalogue of Somatic Mutations in Cancer (COSMIC) database curated by Wellcome Trust Sanger Institute. We then determined the somatic status of each SNV (or indel) by comparing the genotypes and likelihood between matched normal and tumor samples. The somatic status of a specific SNV/indel was reported if the matched normal had wild allele–based homozygous genotype and the tumor had heterozygous or mutant allele–based homozygous genotype with a certain cutoff of genotype likelihood/*P*-value (0.99). Finally, each somatic mutation or indel was annotated with its functional effect by SIFT (http://sift.jcvi.org, J Craig Venter Institute, San Diego, CA) to determine whether a mutation candidate was synonymous or nonsynonymous (benign or deleterious). Five nevus samples and five matched blood samples were processed and analyzed using whole-exome sequencing (Otogenetics, Norcross, GA). Library construction followed the procedure of previous publications (Stransky *et al.*, 2011; Berger *et al.*, 2011; Chapman *et al.*, 2011). Exome sequencing data were then validated by Sanger sequencing and when necessary allele-specific PCR. The allele-specific PCR approach was used to validate the 7 non-*NRAS*, non-*BRAF* mutations using WT and mutant primer couples for each gene. Primer annealing temperature was selected to allow WT allele amplification with WT complementary primers and not with the mutant complementary primer.

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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