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Whole exome sequencing reveals somatic mutations in *HRAS* and *KRAS* which cause nevus sebaceus

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TO THE EDITOR

Epidermal genetic mosaicism is evident as stripes of affected skin which typically appear in S or V-shaped whorled, streaked, and linear patterns called lines of Blaschko (Blaschko, 1901). These patterns represent dorsoventral migratory pathways of neuroectoderm during embryogenesis (Moss *et al.*, 1993). Mosaic lesions result from somatic mutation during development, with timing of such events determining the extent and distribution of skin involvement. Epidermal nevi (EN) are common cutaneous mosaic disorders seen in 0.1–0.3% of births, and fall into two classes: keratinocytic epidermal nevi (KEN) and organoid epidermal nevi, which includes nevus sebaceus (NS) and follicular nevi (Solomon and Esterly, 1975). NS comprises approximately half of EN, and typically appears on the scalp as a yellowish-orange linear plaque with hyperkeratosis, acanthosis, a markedly increased number of sebaceous lobules and abortive hair follicles with resulting alopecia (Figure 1a–

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Conflict of Interest

The authors report no conflict of interest.

d). In contrast to KEN, in which neoplasia is rare, tumors develop in nearly 14% of all NS cases, and in more than 23% of affected adults (Cribier *et al.*, 2000), suggesting that the mutation(s) causing NS also increase risk of tumorigenesis (Figure 1e–g).

Recently, somatic mosaicism has been identified in KEN using SNaPshot assays to identify mutations in MAPK pathway genes including *FGFR3*, *HRAS*, *KRAS*, *NRAS* and *PIK3CA*. Activating Ras mutations, including *HRAS* p.Gly13Arg and *KRAS* p.Gly12Asp, were the most common and accounted for 39% of KEN, with *HRAS* mutations predominating (Hafner *et al.*, 2012). Similar approaches have been employed in NS, identifying *HRAS* p.Gly13Arg in 91% of lesions and *KRAS* p.Gly12Asp in 5% of lesions (Groesser *et al.*, 2012). We present an independent, complementary approach to genetic pathogenesis in NS in which we employed whole exome sequencing to characterize the spectrum of *de novo* coding mutations present within NS lesions.

For our study cohort, we identified five individuals with nevus sebaceus and performed exome sequencing of paired DNA from blood and NS tissue in each (Supplementary Table 1). Genetic variants were annotated and compared to identify *de novo* somatic mutations present solely within nevus sebaceus tissue and not in germline DNA. Via this analysis, we identified two genes with recurrent somatic mutations: three NS samples had an identical somatic mutation in *HRAS* (p.Gly13Arg), and the remaining two had a somatic mutation at the Gly12 residue of *KRAS* (p.Gly12Asp and p.Gly12Val, respectively) (Figure 2, Supplementary Figure 1). Furthermore, no genes other than *HRAS* and *KRAS* were found to be mutated in more than one lesion. Notably, one sample showed a concurrent *HRAS* p.Gly13Arg and a *BRAF* p.Arg347Gln mutation which has not previously been described as a germline or somatic mutation. This *BRAF* mutation was the only other somatic mutation found in exome sequences of these five NS samples.

To examine whether these mutations were present uniquely in the epidermis, and not in the underlying dermis, we used laser capture microdissection to prepare DNA independently from epidermis and dermis of NS lesions and then performed Sanger sequencing with *HRAS*- or *KRAS*-specific primers. We found the mutant allele in an approximately equimolar ratio in epidermis and sebaceous lobules, but it was entirely absent in dermis and blood (Supplementary Figures 2 and 3). Via Sanger sequencing, we evaluated 11 additional paired samples for *HRAS* and *KRAS* mutations, and all were found to have the same somatic p.Gly13Arg *HRAS* mutation (Supplementary Table 2; Supplementary Figure 3).

Prior studies have reported that up to 40% of NS lesions exhibit loss of heterozygosity (LOH) on chromosome 9q, inclusive of *PTCH* (Xin *et al.*, 1999). Examination of exome data from our discovery cohort found no evidence of LOH in NS lesions at this locus or elsewhere (Supplementary Figure 4).

Recognizing that *HRAS* and *KRAS* are oncogenes, that observed mutations could serve as an initiating event in multistep carcinogenesis (Knudson, 2001), and that tumors frequently develop in NS, we sought to determine if tumors arise specifically in *HRAS* or *KRAS* mutation-positive lesions. Neoplasms arising within NS are typically benign and consist primarily of trichoblastomas, syringocystadenoma papilliferum, trichilemmomas, and

tubular apocrine adenomas (Cribier *et al.*, 2000), although occasional basal cell carcinomas and rarely more aggressive malignant tumors have been reported (Moody *et al.*, 2012). We identified 11 archival NS specimens containing tumors, isolated DNA from the NS portion of the lesion, and found that all samples had an *HRAS* p.Gly13Arg mutation (Supplementary Table 2, Supplementary Figure 5). The spectrum of additional genetic events necessary for tumorigenesis in NS lesions remains unknown.

In total, 27 nevus sebaceus samples were evaluated, with 25 harboring an identical *HRAS* mutation (p.Gly13Arg), and two exhibiting a *KRAS* mutation in the adjacent paralogous residue (p.Gly12Asp or p.Gly12Val). The occurrence of multiple, tightly clustered somatic mutations in adjacent residues of these highly homologous proteins is definitive proof of a role for these mutations in nevus sebaceus and suggests a gain of function mechanism. Indeed, expression of the *KRAS* p.Gly12Asp allele within murine hair follicles reproduces features of NS including abortive hair follicles and epidermal and sebaceous gland hyperplasia (Lapouge *et al.*, 2011; Mukhopadhyay *et al.*, 2011).

Missense mutations at codons 12 and 13 of *HRAS* and *KRAS*, respectively, are common in malignancies, including squamous cell carcinoma, and lead to constitutive activation of Ras by blocking the activity of GTPase activating proteins (Grewal *et al.*, 2011). Ras isoforms are central regulators of the mitogen-activated protein kinase (MAPK) pathway which controls cell proliferation, differentiation and survival. Rare inherited disorders caused by germline mutations in Ras and other MAPK pathway members, known as “RASopathies,” include Costello, Noonan, and cardio-facio-cutaneous syndrome. These show variable cutaneous features, particularly hyperkeratosis, palmoplantar keratoderma, papillomas, and hair abnormalities in addition to craniofacial and systemic findings. KEN and NS have not been reported in affected individuals. Notably, *HRAS* p.Gly13Arg and *KRAS* p.Gly12Asp have not been reported as germline mutations in these or other disorders, and *KRAS* p.Gly12Asp leads to embryonic lethality in mice, suggesting that both mutations grossly disrupt embryonic development and are thus likely to be found primarily in mosaic states (Tuveson *et al.*, 2004). Our findings confirm the predominance of *HRAS* mutations in NS, including those with tumors (Groesser *et al.*, 2012), and provide evidence that *HRAS* and *KRAS* mutations are sufficient to cause NS without genome instability, LOH, or secondary mutation.

The marked sebaceous hyperplasia observed NS, which are found almost exclusively on the scalp and face, is not seen in KEN, which appear primarily on the torso, despite identical underlying somatic *HRAS* and *KRAS* mutations. This suggests that body site determines phenotype and is supported by a report of a contiguous linear nevoid lesion extending from the scalp to the neck with transition in clinical and histologic appearance from KEN on the upper back and neck to NS on the scalp (Waltz *et al.*, 1999). The specific determinants of such site-specific phenotypes are unknown, though distinct epithelial-mesenchymal interactions are a possible cause.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

EN	epidermal nevi
KEN	keratinocytic epidermal nevi
NS	nevus sebaceus
SNV	single nucleotide variation
SNP	single nucleotide polymorphism
LOH	loss of heterozygosity
SCP	syringocystadenoma papilliferum
TAA	tubular apocrine adenoma
TB	trichoblastoma
TL	trichilemmoma

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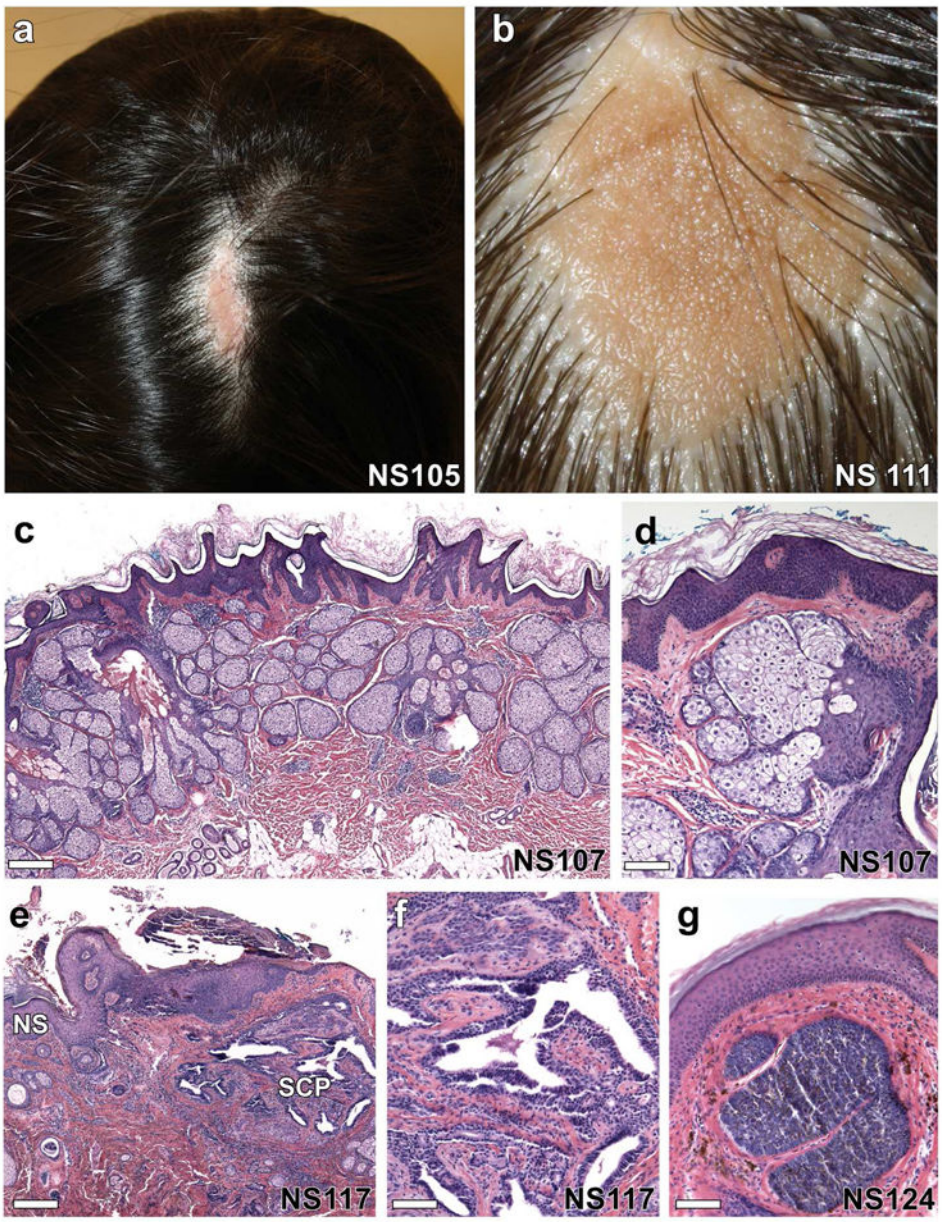

Figure 1. Clinical and microscopic features of nevus sebaceus

(**a, b**) Solitary, well-demarcated lesions on the scalp of two individuals show alopecia and a yellow-orange waxy appearance. (**c**) On histological examination, there is epidermal acanthosis, papillomatosis, and hyperkeratosis with dramatic increase in the number of sebaceous lobules and abortive hair follicles, scale = 288 μm, which is more evident at higher magnification (**d**), scale = 85 μm. (**e–g**) Up to 20% of nevus sebaceus lesions develop tumors including syringocystadenomas, trichoblastomas, trichilemmomas and tubular apocrine adenomas. (**e**) Nevus sebaceus (NS) with syringocystadenoma papilliferum (SCP) composed of villous structures lined by a columnar epithelium with stromal plasma cells, scale = 570 μm, most evident at higher magnification, (**f**), scale = 92 μm. (**g**) A

trichoblastoma arising within a nevus sebaceus shows a well-circumscribed nodule of basaloid cells with a dense fibrocytic stroma, scale = 92 μm.

a

Sample	Position (hg18)	Gene	Base change	Protein Effect	# reads-tissue Ref.	# reads-tissue Non-ref.	# reads-blood Ref.	# reads-blood Non-ref.	P-value
NS101	Chr11:524,286	***HRAS***	G>C	**G13R**	96	17	70	1	$1.2x10^{-3}$
NS102	Chr11:524,286	***HRAS***	G>C	**G13R**	212	62	123	0	$8.9x10^{-12}$
NS103	Chr11:524,286	***HRAS***	G>C	**G13R**	135	19	141	0	$2.5x10^{-6}$
NS107	Chr12:25,289,551	***KRAS***	G>T	**G12V**	202	62	102	0	$1.6x10^{-10}$
NS109	Chr12:25,289,551	***KRAS***	G>A	**G12D**	154	31	77	0	$9.1x10^{-6}$

b

Figure 2. Exome sequencing reveals somatic *HRAS* and *KRAS* mutations in nevus sebaceus tissue

(**a**) *HRAS* and *KRAS* mutation annotation, including genomic position, nucleotide change, protein consequence, and number of reference and non-reference reads obtained from paired sequencing of tissue and blood in 5 independent, unrelated nevus sebaceus cases. Significance of the mutant allele frequency difference between tissue and blood DNA was calculated with a one-tailed Fisher's exact test. When corrected for multiple testing, 2.4×10^{-6} is the threshold for genome wide significance. In each case, *HRAS* and *KRAS* mutations showed the lowest P-value. (**b**) Alignment of the N-termini of HRAS and KRAS reveals identical residues through position 94, with an overall 95% identity and 99% similarity. The first 50 amino acids are shown for the wild-type and each mutant protein, with mutant residues indicated in red.