SHORT REPORT

Keratinocytic epidermal nevi are associated with mosaic *RAS* mutations

Christian Hafner,[1] Agusti Toll,[2] Susanne Gantner,[1] Andreas Mauerer,[1] Irene Lurkin,[3] Francesco Acquadro,[4] Alejandro Fernández-Casado,[2] Ellen C Zwarthoff,[3] Wolfgang Dietmaier,[5] Eulalia Baselga,[6] Elisabet Parera,[2] Asunción Vicente,[7] Ariel Casanova,[8] Juan Cigudosa,[4] Thomas Mentzel,[9] Ramon M Pujol,[2] Michael Landthaler,[1] Francisco X Real[8,10]

[1]Department of Dermatology, University of Regensburg, Regensburg, Germany
[2]Servei de Dermatologia, Hospital del Mar-Parc de Salut Mar, Universitat Autònoma de Barcelona, Barcelona, Spain
[3]Department of Pathology, Josephine Nefkens Institute, Erasmus MC, Rotterdam, The Netherlands
[4]Grupo de Citogenética Molecular and CIBERER, Programa de Genética del Cáncer Humano, Centro Nacional de Investigaciones Oncológicas, Madrid, Spain
[5]Institute of Pathology, University of Regensburg, Regensburg, Germany
[6]Department of Dermatology, Hospital de la Santa Creu i Sant Pau, Barcelona, Spain
[7]Department of Dermatology, Hospital Sant Joan de Déu, University of Barcelona, Spain
[8]Grupo de Carcinogénesis Epitelial, Programa de Patología Molecular, Centro Nacional de Investigaciones Oncológicas, Madrid, Spain
[9]Dermatopathology, Friedrichshafen, Germany
[10]Departament de Ciències Experimentals i de la Salut, Universitat Pompeu Fabra, Barcelona, Spain

Correspondence to
Dr Christian Hafner, Department of Dermatology, University of Regensburg, Franz-Josef-Strauss-Allee 11, 93053 Regensburg, Germany; christian.hafner@ukr.de

CH and AT contributed equally to this study.

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ABSTRACT

Background Activating *RAS* mutations in the germline cause rare developmental disorders such as Costello syndrome. Somatic *RAS* mutations are found in approximately 30% of human cancers. Keratinocytic epidermal nevi (KEN) represent benign congenital skin lesions arranged along Blaschko's lines. A subgroup of KEN is caused by hotspot oncogenic *FGFR3* and *PIK3CA* mutations in mosaicism, but the majority lack these mutations.

Methods This study screened 72 KEN for activating mutations in *RAS* genes and other oncogenes.

Results Activating *RAS* mutations were identified in 28/72 (39%) of KEN. *HRAS* was the most commonly affected oncogene (86%), with the *HRAS* p.G13R substitution representing a new hotspot mutation.

Conclusion These results indicate that activating *RAS* somatic mutations leading to mosaicism result in benign KEN of the skin. Given the prevalence of KEN, mosaic *HRAS* mutations appear to be more common in patients than germline ones. These findings identify KEN as a mosaic RASopathy and lend further support to the notion that genetic mosaicism is an important contributor to disease.

INTRODUCTION

Ras proteins regulate cell proliferation, survival, and differentiation. Somatic *RAS* mutations resulting in constitutive active proteins occur in 30% of human tumours; progression models assume that these mutations occur in the adult.[1] Activating germline *RAS* mutations have been identified in rare developmental disorders, such as *HRAS* mutations in Costello syndrome (CS), characterised by prenatal overgrowth and postnatal growth restriction, coarse face, skin alterations, cardiomyopathy, and cancer predisposition.[2 3] CS overlaps with Noonan and cardio-facio-cutaneous syndromes, caused by activating mutations in other genes of the RAS–RAF–MAPK pathway.[4–6]

Non-organoid keratinocytic epidermal nevi (KEN) are benign congenital lesions displaying a linear distribution and resulting from genetic mosaicism; approximately 40% of KEN harbour postzygotic activating mutations in *FGFR3* and *PIK3CA* genes.[7 8] However, the underlying gene mutation in the majority of KEN still remains unknown. In this study we show that approximately 40% of KEN are caused by postzygotic activating *RAS* mutations displaying a strong association between the genotype and the phenotype, with a predominance of the *HRAS* p.G13R mutation.

MATERIALS AND METHODS

Sample acquisition

To identify novel genes causing KEN, we screened one lesion from each of 72 patients for hotspot mutations in various genes (table 1). From one patient (no.27), three different biopsies of the KEN were available for analysis. The KEN were retrieved from the histopathological files of the contributing departments in Regensburg, Barcelona, and Friedrichshafen. The male:female ratio of the patients was 1:1.1, and the mean age at the time of the biopsy was 18.9±13.1 years (table 1). The study was approved by the local ethics committees of the participating institutions and was performed according to the Declaration of Helsinki.

Mutation analyses

DNA was isolated from manually microdissected sections of formalin-fixed, paraffin-embedded tumours. *FGFR3*, *PIK3CA*, *HRAS*, *NRAS*, and *KRAS* mutations were analysed using SNaPshot assays (Applied Biosystems, Carlsbad, California, USA) as described previously.[7–10] Each mutation was confirmed by a second independent PCR. Exon 4 of *AKT1* harbouring the p.E17K hotspot mutation was sequenced directly as described previously.[11] Furthermore, direct sequencing (*NRAS* and *HRAS*) and pyrosequencing (*KRAS*) were performed in a subset of samples for validation of the mutations in independent laboratories. To screen for mutations in other oncogenes, the OncoCarta Panel v1.0 (Sequenom, San Diego, California, USA) covering mutations in *ABL1*, *AKT1*, *AKT2*, *BRAF*, *CDK4*, *EGFR*, *ERBB2*, *FGFR1*, *FGFR3*, *FLT3*, *HRAS*, *JAK2*, *KIT*, *KRAS*, *MET*, *NRAS*, *PDGFRA*, *PIK3CA*, and *RET* was used for a subset of 10 KEN being wild-type for the mutations mentioned above.[12]

RESULTS

Activating *FGFR3* and *PIK3CA* mutations were found in 16/72 (22%) and in 6/72 (8%) KEN, respectively; hotspot oncogenic *RAS* mutations were identified in 28/72 (39%) of KEN (figure 1A;

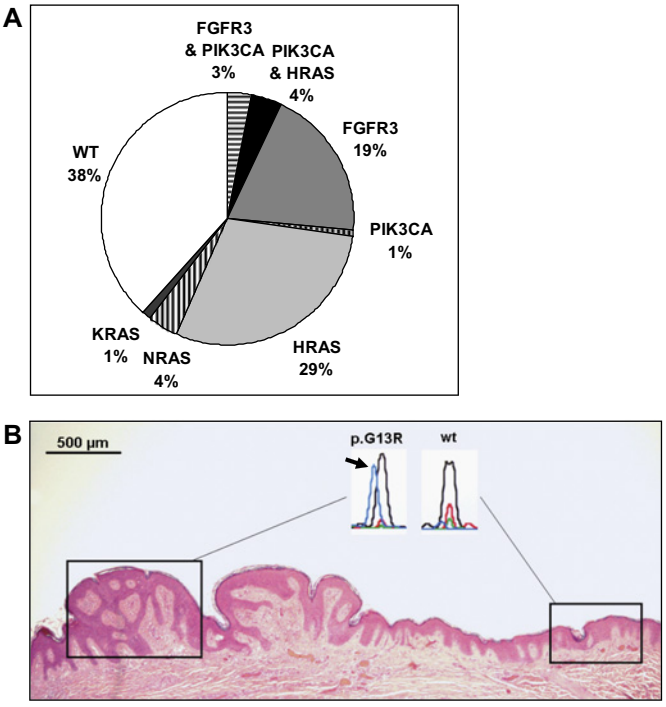

Figure 1 Mutational analysis of keratinocytic epidermal nevi. (A) Mutation distribution (WT, wildtype). (B) The *HRAS* p.G13R mutation is present in the epidermal nevus but absent from normal epidermis, indicating a strong association between the mutation and the epidermal hyperplasia.

table 1). *HRAS* was the most frequently altered gene: in 21/24 *HRAS*-mutant lesions, the p.G13R (c.37G>C) substitution was found, thus representing a new hotspot mutation in KEN. In one patient, this mutation was found in three biopsies at different sites of the KEN. According to the COSMIC database (http://www.sanger.ac.uk/genetics/CGP/cosmic/), the p.G13R mutation is the most common *HRAS* mutation at codon 13 and has been described in tumours of the thyroid gland, upper aerodigestive tract, and urinary tract. The remaining three *HRAS* mutations (p.G12C, p.G12V, and p.Q61L) have also been previously described as somatic mutations in human tumours. Normal epidermis (n=4) and dermis (n=3) adjacent to the KEN, as well as blood DNA (n=2), were available from some patients whose KEN had a *HRAS* p.G13R mutation. These normal tissues displayed a wild type *HRAS* sequence (figure 1B), indicating the somatic nature of the mutation and a strong association between its presence and the clinical phenotype of KEN. Three KEN harboured mutations in *NRAS* (p.G12D, p.P34L, and p.Q61R) and one in *KRAS* (p.G12D). *AKT1* mutations were absent from 47 KEN analysed. In five KEN, two mutations were found simultaneously: *HRAS* (n=3) or *FGFR3* (n=2) mutations co-occurred with *PIK3CA* mutations, but *HRAS* and *FGFR3* mutations were mutually exclusive, as reported previously in cancers.[13] Similar mutational patterns are associated with seborrhoeic keratoses, benign epidermal tumours of the adult.[7 11] Overall, no mutation was identified in 27/72 (38%) KEN. To screen for further oncogenic mutations, 10 of these wildtype KEN were analysed using the OncoCarta Panel that covers 238 mutations in 19 oncogenes.[12] However, no additional mutations were identified using this technique.

DISCUSSION

Despite much research on *RAS*, there is still incomplete knowledge of the biological effects resulting from distinct *RAS* gene mutations. In the last few years, the concept of 'RASopathy' has been applied to developmental syndromes caused by germline mutations in genes of the RAS/MAPK pathway.[14 15] The RASopathies comprise CS, Noonan syndrome, LEOPARD syndrome (**l**entigines, **E**CG conduction abnormalities, **o**cular hypertelorism, **p**ulmonic stenosis, **a**bnormal genitalia, **r**etardation of growth, and sensorineural **d**eafness), cardio-facio-cutaneous syndrome, neurofibromatosis type 1, hereditary gingival fibrosis, capillary malformation–arteriovenous malformation syndrome, autoimmune proliferative syndrome, and Legius syndrome. The involvement of a common signalling pathway explains the phenotypical overlap between many of these disorders.

In this study we report that postzygotic *RAS* mutations affecting the epidermis can cause KEN. *RAS* genes therefore join *FGFR3* and *PIK3CA* as oncogenes involved in benign congenital proliferative skin lesions. In KEN, *HRAS* was the most frequently mutated *RAS* gene. Germline *HRAS* mutations cause CS affecting a wide variety of tissues, including the skin, and involving both the epidermis and the dermis. Typical dermatological findings in CS patients comprise papillomas, palmoplantar keratoderma, deep palmar and plantar creases with loose and redundant skin, pachydermatoglyphia, abnormal fingernails, curly or wavy hair, generalised hyperpigmentation, hyperpigmented patches, and acanthosis nigricans.[16] The latter is also found in patients with *FGFR3* germline mutations and shows histopathological similarities to KEN. Intriguingly, germline mutations of *FGFR3* and *HRAS* are associated with acanthosis nigricans, whereas somatic postzygotic mutations of both genes in the skin result in KEN.

Mosaicism affecting *RAS* genes has been reported in a small number of subjects. Two patients with a CS phenotype harboured a p.G12S *HRAS* mosaicism,[17] suggesting an early occurrence of the mutation with extensive contribution to adult tissues. The sharply demarcated plaques and papules following Blaschko's lines are characteristic of KEN but have not been described in classical CS, indicating that *HRAS* mutant KEN are not simply incomplete manifestations of CS. Based on the clinical data available, none of the patients included in this study had typical features of CS or related disorders.

The mechanisms for the distinct phenotypes associated with germline versus postzygotic *RAS* mutations are unknown. Depending on the developmental stage and fate potential of the cells in which the mutations occur, different tissue compartments and cell types will be affected (eg, epidermis only versus both epidermis and dermis). Mosaicism implies the coexistence of diverse cell populations within a given tissue, with potential population cross-talk effects. Our findings suggest that in KEN mutations occur in a subset of epidermal precursors. Differences in the spectrum found in mosaic versus germline mutations could also contribute to the distinct phenotype. For example, p.G13R is the most common *HRAS* mutation in KEN whereas p.G12S is characteristic of CS. Differences in the mutational spectrum may, at least in part, be due to the strength of the biological effects of the amino acid substitution and the associated potential embryonic lethality. Similarly, the spectrum of *KRAS* mutations found in the germline in patients with developmental disorders differs from that of somatic changes in patients with cancer.[4] Interestingly, the *HRAS* p.G13R mutation occurs in tumours as well as in KEN, but it has not been described in the germline of CS patients, suggesting that although it is tolerated in the mosaic state, it may be lethal in the germline. The *KRAS* p.G12D mutation—the most frequent somatic *KRAS* substitution found in cancer—has been described in the mosaic

Table 1 Mutation analysis of keratinocytic epidermal nevi

No.	Sex	Age	Localization	*FGFR3*	*PIK3CA*	*KRAS*	*HRAS*	*NRAS*	*AKT1*	Normal control tissue
1	M	23	Head	wt	E542K	wt	G13R	wt	wt	
2	F	9	Trunk right	R248C	NA	wt	wt	wt	wt	
3	M	15	Head	wt	NA	NA	G12C	wt	wt	wt (dermis)
4	M	17	NA	wt	wt	wt	wt	wt	wt	
5	M	9	Neck right	R248C	wt	wt	wt	wt	wt	
6	F	16	Arm right	wt	wt	wt	wt	wt	NA	
7	M	21	Head right	wt	wt	NA	G13R	wt	wt	*HRAS* wt (epidermis)
8	F	9	Head	wt	wt	wt	wt	wt	wt	
9	M	23	Head	wt	E542K	wt	G13R	wt	wt	
10	F	18	Lower leg	wt	wt	wt	wt	wt	wt	
11	M	15	Neck left	wt	wt	wt	G13R	wt	wt	*HRAS* wt (blood)
12	F	16	Trunk	wt	wt	wt	G13R	wt	wt	
13	F	16	Neck	wt	wt	wt	wt	wt	wt	
14	M	10	Neck	G372C	wt	wt	wt	wt	R41R (CGG > CGA)	
15	F	17	Trunk	wt	wt	wt	Q61L	wt	wt	
16	M	23	Trunk	wt	wt	wt	G13R	wt	wt	
17	M	25	NA	wt	wt	wt	G13R	wt	wt	
18	F	1	Arm	wt	wt	wt	wt	wt	wt	
19	M	13	Head	wt	wt	wt	wt	wt	wt	
20	M	15	Head	wt	wt	wt	wt	wt	wt	
21	M	16	Neck	wt	wt	NA	wt	wt	wt	
22	F	22	Trunk	wt	wt	wt	wt	wt	wt	
23	M	16	Trunk	wt	wt	G12D	wt	wt	wt	
24	F	14	Neck	wt	wt	wt	G13R	wt	wt	
25	M	11	Trunk	wt	wt	wt	wt	wt	wt	
26	F	7	Head	wt	wt	NA	wt	wt	NA	
27a	M	12	Head	NA	wt	wt	G13R	wt	wt	*HRAS* wt (dermis)
27b	M	12	Head	NA	NA	NA	G13R	NA	NA	
27c	M	12	Head	NA	NA	NA	G13R	NA	NA	*HRAS* wt (epidermis), *HRAS* wt (dermis)
28	F	9	Trunk	R248C	wt	NA	NA	NA	wt	
29	M	10	Trunk	S249C	wt	wt	wt	wt	wt	
30	F	13	Head	wt	wt	wt	wt	wt	wt	
31	F	6	Arm	wt	wt	wt	wt	wt	wt	
32	F	1	Head	wt	wt	wt	wt	NA	NA	
33	F	37	Arm right	R248C	NA	wt	wt	wt	NA	
34	M	18	Head	wt	wt	wt	wt	wt	NA	
35	M	7	Head	wt	wt	wt	wt	wt	NA	
36	M	21	Trunk left	R248C	wt	wt	wt	wt	NA	
37	M	30	Head	R248C	E545K	wt	wt	wt	NA	
38	F	34	Trunk	wt	wt	wt	G13R	wt	NA	*HRAS* wt (epidermis)
39	F	2	Neck	wt	wt	wt	G13R	wt	NA	*HRAS* wt (epidermis), wt (blood)
40	M	6	Neck	R248C	NA	wt	wt	wt	NA	
41	M	16	Head right	wt	wt	wt	wt	NA	NA	
42	F	28	Neck	wt	NA	wt	wt	wt	NA	
43	F	25	Trunk	wt	NA	wt	G13R	wt	NA	
44	F	14	Trunk	wt	wt	wt	G13R	wt	NA	
45	F	16	Trunk	wt	NA	wt	wt	wt	NA	
46	F	21	Arm right	R248C	NA	wt	wt	wt	NA	
47	M	31	Head	wt	NA	NA	wt	wt	NA	
48	F	19	Trunk	wt	NA	wt	wt	wt	NA	
49	F	12	Arm left	R248C	NA	wt	wt	wt	NA	
50	M	23	Trunk	R248C	wt	wt	wt	wt	NA	
51	F	14	Neck	R248C	NA	wt	wt	wt	NA	
52	F	14	Head	wt	E545G	wt	G13R	wt	wt	
53	M	18	Trunk	R248C	E545G	wt	wt	wt	wt	
54	M	2	Trunk	wt	H1047R	wt	wt	wt	wt	
55	F	8	Head	wt	wt	wt	wt	wt	wt	
56	M	10	Head	R248C	wt	wt	wt	wt	wt	
57	M	70	Neck (sys)	wt	wt	wt	G12V	wt	wt	
58	M	0	Arm (sys)	wt	wt	wt	wt	wt	wt	

Continued

Table 1 Continued

No.	Sex	Age	Localization	*FGFR3*	*PIK3CA*	*KRAS*	*HRAS*	*NRAS*	*AKT1*	Normal control tissue
59	M	12	Trunk	wt	wt	wt	G13R	wt	wt	
60	F	50	Trunk	wt	wt	wt	G13R	wt	wt	
61	F	29	Head	wt	wt	wt	G13R	wt	wt	
62	M	18	Trunk	wt	wt	wt	G13R	wt	wt	
63	F	14	Head	wt	wt	wt	G13R	wt	wt	
64	M	23	Head	wt	wt	wt	wt	Q61R	wt	
65	F	12	Neck	R248C	wt	wt	wt	wt	wt	
66	F	27	Trunk	wt	wt	wt	wt	wt	wt	
67	F	64	Arm	wt	wt	wt	G13R	wt	wt	
68	F	45	Head	wt	wt	wt	wt	P34L	NA	
69	F	39	Trunk	wt	wt	wt	G13R	wt	NA	
70	F	15	Trunk	wt	wt	wt	wt	wt	wt	
71	F	46	Neck	wt	wt	NA	NA	G12D	NA	
72	F	12	Trunk	wt	wt	wt	wt	wt	wt	

Age, age at the time of the biopsy (years); f, female; M, male; NA, not available; sys, systematized epidermal nevus; wt, wildtype.

state in a 6-month-old infant with a KEN and a rhabdomyosarcoma[18] and is reported here in one KEN. This amino acid substitution has not been described in the germline, in agreement with the fact that its constitutive expression in the mouse is embryonic lethal.[19] Again, these data suggest that *KRAS* p.G12D is compatible with development and life only when it occurs in the mosaic state. Genetic mouse models allowing the controlled spatial and temporal activation of expression of *RAS* mutants in mice are potent tools to assess these hypotheses experimentally.

KEN occur in approximately 0.1% of live births[20] and about one third of them harbour *HRAS* mutations. By contrast, CS is very rare with only approximately 200 cases having been identified worldwide and a birth prevalence in the UK of approximately 1/500 000.[15] Based on these numbers, postzygotic *HRAS* mutations leading to mosaicism must be considerably more prevalent in patients than germline mutations. The common occurrence of mutations in oncogenes in KEN raises important questions regarding the mechanism of tumour formation and the risk of cancer in affected individuals. Secondary benign or malignant tumours have only seldom been reported in association with KEN, which are generally considered to be devoid of malignant potential. However, the risk of cancer associated with postzygotic mutations might depend on the extent of mosaicism, the tissues or cell types involved, and the specific mutation. *HRAS*, *KRAS*, and *NRAS* are involved in human tumours with a highly tissue-specific distribution that is not simply related to tissue expression patterns. Somatic *HRAS* mutations are common in tumours from the skin, cervix, upper aerodigestive tract, urinary tract, and salivary gland according to the COSMIC database.

In CS, *HRAS* germline mutations are associated with a predisposition to rhabdomyosarcoma, neuroblastoma, and urothelial carcinoma.[21] An association between KEN and noncutaneous malignancies of various organs has been reported, but the incidence and the individual risk for KEN patients is not known.[22] Although the occurrence of tumours in the patients with KEN in this study is not known, it is conceivable that patients with extensive KEN might have an increased risk of internal malignancies both during childhood and in adults. We have recently reported a subject with an extensive mosaicism for the *HRAS* p.G12S mutation: the patient had a systematised KEN and developed multiple urothelial carcinomas but did not show classical signs of CS.[23] This finding implies that oncogenic *RAS* mutations found in adult solid tumours can occur already during embryogenesis. In addition to the skin involvement, the occurrence of urothelial carcinoma and rhabdomyosarcoma may indicate some degree of phenotypic overlap between CS and KEN syndrome.

Mosaicism of oncogenic mutations can cause morphologically distinguishable lesions such as KEN in the skin, as we show here, but it may also occur in other tissues that are not amenable to simple tissue inspection, and contribute to cancer and non-neoplastic disorders at these sites. The frequency of mosaicism for Ras/MAPK pathway gene mutations in internal organs and tissues is unknown but it could be of clinical relevance.

Our findings suggest that a proportion of KEN can be considered as a RASopathy due to activation of the RAS/MAPK pathway by mutant RAS proteins. Because the term 'RASopathy' is defined by syndromes resulting from germline mutations, we suggest the term 'mosaic RASopathy' to describe KEN and KEN syndrome[18 23] as well as some rare cases of mosaic CS.[17] Additional syndromes may be added to this list in the future because the genetic basis of many mosaic disorders remains unknown.

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Contributors CH designed the study, collected study material and information, supervised experimental work, performed mutation analyses and interpretation of the data, obtained financial support and wrote the manuscript; AT participated in the study design, collected study material and information, data analysis and discussion, and contributed to manuscript writing; SG collected study material and information, and discussed the findings; AM collected study material and information, and discussed the findings; IL developed the SNaPshot assay, contributed to the discussion; FA obtained and analysed data, and discussed the findings; EZ developed the SNaPshot assay, contributed to the discussion; WD performed pyrosequencing KRAS mutation analysis and contributed to the discussion; EB collected study material and information and contributed to the discussion; EP collected study material and information and contributed to the discussion; AV collected study material and information and contributed to the discussion; AC performed mutational analysis, and discussed the findings; JC analysed data and discussed the findings; TM collected study material and contributed to the discussion; RP participated in the study design, discussion, and obtained financial support; ML participated in the study design and the discussion; FXR designed the study, supervised the overall conduct of the study, contributed to the discussion and data interpretation, obtained financial support, and wrote the manuscript with CH. All authors discussed and approved the final version of the manuscript.

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Competing interests None.

Ethics approval Ethics approval was provided by ethical boards of the universities of Regensburg and Barcelona.

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Christian Hafner, Agusti Toll, Susanne Gantner, Andreas Mauerer, Irene Lurkin, Francesco Acquadro, Alejandro Fernández-Casado, Ellen C Zwarthoff, Wolfgang Dietmaier, Eulalia Baselga, Elisabet Parera, Asunción Vicente, Ariel Casanova, Juan Cigudosa, Thomas Mentzel, Ramon M Pujol, Michael Landthaler and Francisco X Real

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