

# Oncogenic *PIK3CA* mutations occur in epidermal nevi and seborrheic keratoses with a characteristic mutation pattern

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Activating mutations of the p110  $\alpha$  subunit of PI3K (*PIK3CA*) oncogene have been identified in a broad spectrum of malignant tumors. However, their role in benign or preneoplastic conditions is unknown. Activating FGF receptor 3 (*FGFR3*) mutations are common in benign skin lesions, either as embryonic mutations in epidermal nevi (EN) or as somatic mutations in seborrheic keratoses (SK). *FGFR3* mutations are also common in low-grade malignant bladder tumors, where they often occur in association with *PIK3CA* mutations. Therefore, we examined exons 9 and 20 of *PIK3CA* and *FGFR3* hotspot mutations in EN ( $n = 33$ ) and SK ( $n = 62$ ), two proliferative skin lesions lacking malignant potential. Nine of 33 (27%) EN harbored *PIK3CA* mutations; all cases showed the E545G substitution, which is uncommon in cancers. In EN, R248C was the only *FGFR3* mutation identified. By contrast, 10 of 62 (16%) SK revealed the typical cancer-associated *PIK3CA* mutations E542K, E545K, and H1047R. The same lesions displayed a wide range of *FGFR3* mutations. Corresponding unaffected tissue was available for four EN and two mutant SK: all control samples displayed a WT sequence, confirming the somatic nature of the mutations found in lesional tissue. Forty of 95 (42%) lesions showed at least one mutation in either gene. *PIK3CA* and *FGFR3* mutations displayed an independent distribution; 5/95 lesions harbored mutations in both genes. Our findings suggest that, in addition to their role in cancer, oncogenic *PIK3CA* mutations contribute to the pathogenesis of skin tumors lacking malignant potential. The remarkable genotype–phenotype correlation as observed in this study points to a distinct etiopathogenesis of the mutations in keratinocytes occurring either during fetal development or in adult life.

oncogene | senescence | skin | benign tumor

Phosphatidylinositol 3-kinases (PI3Ks) are involved in the generation of the second-messenger phosphatidylinositol-3,4,5-trisphosphate (PIP<sub>3</sub>). Three classes of PI3Ks are known. Class I PI3Ks are composed of a regulatory (p85) and a catalytic (p110) subunit. The Class IA subgroup proteins are involved in signaling from receptor tyrosine kinases upon growth factor binding, whereas class IB proteins signal from G-coupled receptors (1). The levels of PIP<sub>3</sub> are also controlled by phosphatase and tensin homologue, a classical tumor suppressor protein with lipid phosphatase activity. PIP<sub>3</sub> generated by the p110 catalytic subunit of class I PI3Ks is anchored to the cell membrane, leading to the binding of PKD through its pleckstrin homology domain, its subsequent activation, and the downstream phosphorylation and activation of the survival kinase AKT. The PI3K pathway has been shown to play major roles in cell growth, proliferation, survival, and malignant transformation (2).

Four different p110 subunits ( $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$ ) have been identified, and all of them have transforming potential *in vitro*. Overexpression of WT p110  $\beta$ ,  $\gamma$ , or  $\delta$  is oncogenic; by contrast, p110  $\alpha$  is oncogenic only when constitutively activated through point mutation (3). Indeed, activating somatic mutations have only been reported in the gene coding for the  $\alpha$  isoform, *PIK3CA*, in a wide variety of human cancers, such as breast, colon, ovarian, and gastric cancer (4, 5). Somatic *PIK3CA* point mutations cluster in hotspots of exons 9 (helical domain) and 20 (kinase domain). These mutations result in a constitutive gain of function and confer oncogenic properties to mutated cells by deregulation of transcription and translation (6). The *in vivo* oncogenicity of mutant p110 $\alpha$  carrying the most common hotspot mutations E542K, E545K, and H1047R has been recently demonstrated in a chicken model (7).

Epidermal nevi (EN) and seborrheic keratoses (SK) are keratinocyte-derived benign acanthotic skin tumors that never progress to acquire invasive features (Fig. 1). Both entities share clinical and histological characteristics. However, their natural history is very different. EN are congenital lesions that are present at birth or that develop early during childhood as localized epidermal thickening with hyperpigmentation (Fig. 1A). EN follow frequently the lines of Blaschko and may vary in their extent from a single linear lesion to widespread and systematized involvement. It has been proposed that EN result from mosaicism in the skin (8). By contrast, SK are rare in young people. Their prevalence increases with age, and they can be identified by age 50 in 80–100% of healthy individuals (9), although there is wide variation in the number of lesions. SK present as well demarcated brownish plaques with a verrucous surface predominantly localized in the head, neck, and trunk (Fig. 1B). EN and SK share many histological characteristics

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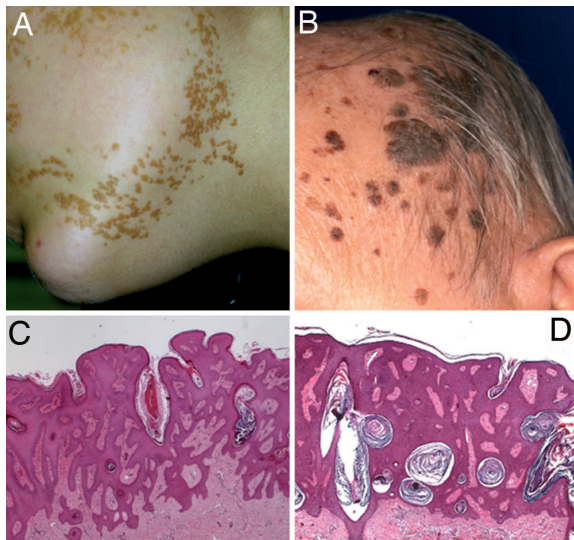
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Abbreviations: EN, epidermal nevus; FGFR3, FGF receptor 3; PI3K, phosphatidylinositol 3-kinase; PIK3CA, p110  $\alpha$  subunit of PI3K; SK, seborrheic keratosis.

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**Fig. 1.** Morphological similarities of linear lesions of congenital EN from a child following Blaschko's lines (A) and SK from an elderly patient (B). At the microscopic level, both lesions are characterized by acanthosis, papillomatosis, and variable degrees of hyperkeratosis and hyperpigmentation (C, EN; D, SK).

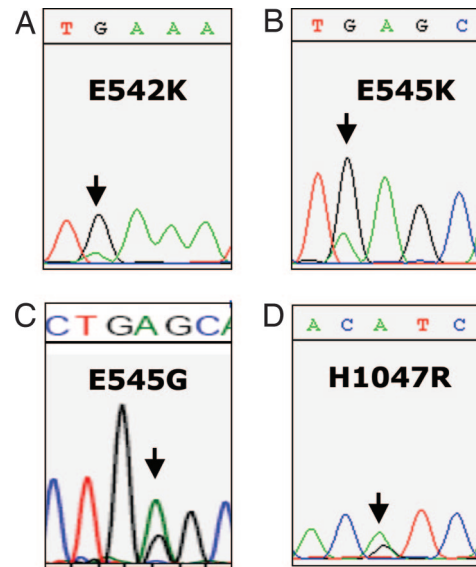
such as acanthosis, papillomatosis, and variable degrees of hyperkeratosis and hyperpigmentation (Fig. 1 C and D).

Recently, activating mutations in the gene coding for FGF receptor 3 (*FGFR3*) were identified in both EN and SK (10, 11). The same mutations detected in these skin lesions have previously been identified as somatic alterations in several types of human cancer (12, 13) as well as in the germ-line in patients with short limb dysplasias (14). The reported *FGFR3* mutations lead to a constitutive ligand-independent activation of the receptor tyrosine kinase activity, and a good correlation between the level of kinase activation and the severity of the skeletal phenotype has been observed (15). Because only  $\approx 30\%$  of EN (10, 16) and 25–85% of SK (11, 17, 18) harbor *FGFR3* mutations, the involvement of additional genes has been postulated (16, 19). There is strong evidence indicating that the PI3K pathway is involved in *FGFR* signaling (20, 21). Therefore, genes in this pathway are potential candidates involved in the pathogenesis of EN and SK. Interestingly, the highest prevalence of *FGFR3* mutations in cancer is found among superficial papillary bladder tumors (22–25). Activating *PIK3CA* mutations have also been reported in superficial papillary bladder tumors, often in association with *FGFR3* mutations (26). These findings suggested that *PIK3CA* mutations might also occur in benign skin tumors. Therefore, we screened EN and SK for *PIK3CA* mutations in exons 9 and 20.

Here, we report that both types of benign acanthotic skin lesions indeed harbor somatic *PIK3CA* mutations in the hotspot codons as previously identified in malignant tumors. In addition, we demonstrate that the mutation spectrum of *PIK3CA* is different in EN and SK, indicating a remarkable genotype-phenotype correlation.

**Table 1. *PIK3CA* mutations in EN and SK**

<i>PIK3CA</i> mutation	Base substitution	Amino acid substitution	<i>n</i> (EN = 33)	<i>n</i> (SK = 62)	Concomitant <i>FGFR3</i> mutation
E542K	c.1624G > A	Glu > Lys	—	7	3 (R248C, K652E)
E545K	c.1633G > A	Glu > Lys	—	2	—
E545G	c.1634A > G	Glu > Gly	9	—	2 (R248C)
H1047R	c.3140A > G	His > Arg	—	1	—



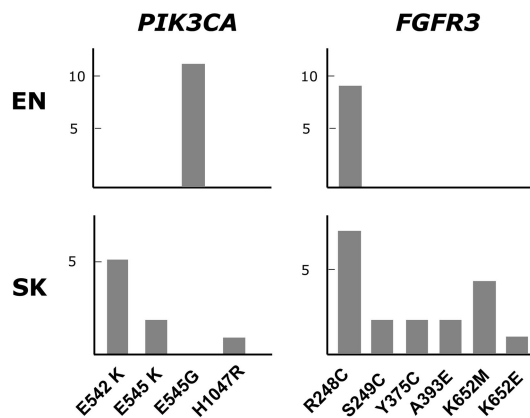
**Fig. 2.** Mutational analysis of EN and SK. In all cases, the mutant sequence was accompanied by the presence of the WT allele, indicating heterozygosity.

## Results

Table 1 summarizes mutational analyses. [Supporting information \(SI\) Tables 2 and 3](#) provide detailed information on the clinical characteristics of the patients and molecular findings.

**Activating *PIK3CA* Mutations Are Common in EN.** We sequenced exons 9 and 20 of *PIK3CA* in 33 EN. The E545G mutation was detected in nine cases (27%) (Fig. 2); no other alterations were found. In all cases, the WT sequence was also present, indicating heterozygosity. Codon 545 is a hotspot for activating mutations in a wide variety of tumors (5). However, in cancer, the E545K mutation is much more common than the E545G mutation, which has been reported only in a few carcinomas (4, 26) ([www.sanger.ac.uk/genetics/CGP/cosmic](http://www.sanger.ac.uk/genetics/CGP/cosmic)). Mutations were not associated with sex, localization, or histological subtype. It has been proposed that EN result from somatic mutations occurring during development, leading to mosaicism, and evidence supporting this notion has been acquired in the case of *FGFR3* mutations (10, 16). We analyzed six unaffected tissue samples from four patients with EN harboring mutant *PIK3CA* sequences and found that only the WT sequence was detectable. In an additional case, leukocyte DNA also displayed only the WT sequence. These findings provide evidence that *PIK3CA* mutations occur in mosaicism in the skin, analogous to *FGFR3* mutations.

**Activating *PIK3CA* Mutations Occur in SK.** Because of the histological similarities between EN and SK and the recent demonstration that *FGFR3* mutations occur in both types of lesions, we also analyzed exons 9 and 20 of *PIK3CA* in SK. Ten of 62 (16%) lesions analyzed were found to harbor *PIK3CA* mutations. Of them, eight displayed an acanthotic, one a hyperkeratotic, and



**Fig. 3.** Spectrum of mutations found in benign skin lesions in *PIK3CA* and *FGFR3*. The number of lesions with each mutation is indicated in the ordinates. EN display a very homogenous mutational pattern in both genes, whereas, in SK, a wide spectrum of mutations as found in malignant cancers is observed.

one an adenoid histologic differentiation. The following amino acid substitutions were found: E542K ( $n = 7$ ), E545K ( $n = 2$ ), and H1047R ( $n = 1$ ) (Table 1; Fig. 2). Analogous to the EN, all *PIK3CA* mutations detected in the SK were heterozygous. Corresponding normal skin, which was available from two patients with mutant SK, was found to be WT, confirming that *PIK3CA* mutations in SK are somatic events. Furthermore, multiple individual SK from three patients were analyzed (four, six, and eight SK per patient, respectively). In two patients, all SK were WT; in the third case, one of the SK was found to be mutated, whereas the other seven lesions from this patient displayed a WT sequence. *PIK3CA* mutations occurred preferentially in SK of the trunk (80%), but this association was not statistically significant ( $P = 0.29$ ). Mutations were not associated with sex, age, or histologic subtype.

***PIK3CA* Mutations and *FGFR3* Mutations in EN and SK.** Fig. 3 summarizes the mutational spectrum findings. The presence of *FGFR3* mutations in this series of EN, and in a proportion of the SK studied, has been reported (10, 16–18). In seven of nine (78%) EN cases with mutant *PIK3CA* sequences, no mutations in *FGFR3* were identified. In the remaining two cases, the R248C mutation in *FGFR3* was associated with the E545G mutation in *PIK3CA*. Five EN showed the R248C hotspot mutation in the *FGFR3* gene in the absence of detectable *PIK3CA* mutations. Similar to EN, 7 of 10 (70%) SK with a *PIK3CA* mutation did not harbor an additional *FGFR3* mutation at the known hotspots. Nineteen of 62 (31%) SK showed activating *FGFR3* mutations: R248C ( $n = 9$ ), S249C ( $n = 2$ ), Y375C ( $n = 2$ ), A393E ( $n = 2$ ), K652E ( $n = 1$ ), and K652M ( $n = 4$ ), one of them with two mutations. Three SK revealed simultaneous *PIK3CA* and *FGFR3* mutations (in two cases, the E542K/R248C and in one case, the E542K/K652E combination was present). In 16 of 62 (26%) SK, an *FGFR3* mutation was found in the absence of *PIK3CA* alterations.

Taken together, *PIK3CA* and/or *FGFR3* mutations were detected in 14 of 33 (42%) EN and in 26 of 62 (42%) SK in this study. Mutations in both genes showed an independent distribution ( $P = 0.908$ ). In 5 of 95 (5%) of the benign skin lesions analyzed, the simultaneous occurrence of mutations in these two oncogenes was demonstrated.

## Discussion

An improved knowledge of the molecular basis of benign tumors is of fundamental importance to understand why benign tumors, unlike malignant ones, fail to progress and invade. Accordingly,

a great deal of emphasis has been placed recently in investigating the cellular response to oncogenic stress as a defense mechanism against tumor progression.

Activating mutations in several oncogenes involved in malignant human tumors, such as *K-ras* or *B-RAF*, have been shown to occur frequently in benign lesions, such as colorectal adenomas or melanocytic nevi (27–29). These benign proliferative lesions carrying mutations show a variable risk for malignant transformation. In this report, we provide evidence that activating mutations in *PIK3CA* previously identified in a wide variety of human cancers also occur in two types of benign skin lesions that bear no relevant risk of progression to malignancy. These findings favor the notion that some benign lesions without malignant potential are initiated by oncogenic pathways shared with cancer. Why some types of benign lesions have the potential to progress to malignancy whereas others do not remains an important question (see below).

EN and SK are histologically similar but display a different natural history. EN are congenital lesions that have been proposed to result from mosaicism in the skin. Approximately 30% of EN display the R248C *FGFR3* mutation in the absence of alterations in unaffected tissue (10, 16). However, there is no information on the genetic basis of the remaining cases. We show that  $\approx 30\%$  of EN display a specific mutation, E545G, in *PIK3CA*. This mutation has only exceptionally been reported in malignant tumors. By contrast, the E545K substitution in the same codon represents  $\approx 25\%$  of all *PI3KCA* mutations in cancers (4). Gymnopoulos *et al.* (30) have recently compared the activity of mutant *PIK3CA* proteins *in vitro* and have shown that the E545G substitution displays strong transforming activity on chicken embryo fibroblasts, although its effect is lower than that of the more common E542K and E545K substitutions.

The mutations identified in *PIK3CA* and *FGFR3* in EN display a remarkable specificity: regarding the former, only the E545G amino acid substitution was present, and regarding the latter, the R248C mutation was almost exclusively found. This genetic homogeneity is in marked contrast with that reported among malignant tumors for both genes (4, 5, 12, 31). The E545G substitution results from an AT:GC transition, whereas the R248C substitution results from a CG:TA transition, suggesting that the biological nature of the mutation, rather than the mutagenic mechanism, plays a crucial role in the development of EN (32).

Unlike EN, SK display a wide spectrum of mutations in both genes (Fig. 3). Regarding *PIK3CA*, the three most common cancer-associated mutations (E542K, E545K, and H1047R) were found; as of *FGFR3*, the mutational spectrum showed even more diversity, including the R248C, S249C, G372C, S373C, Y375C, A393E, K652M, and K652E substitutions (19). Moreover, we have recently reported that, within a given individual with multiple SK, at least four different *FGFR3* mutations were present in each case (18). The etiological and biological relevance of this genetic heterogeneity is currently unknown. However, it suggests that SK arise as a result of a wider range of mutagenic mechanisms than do EN. These observations may have implications for the understanding of the pathogenesis of other tumors in which genetic alterations have been proposed to occur during development.

Most of the mutations in *PIK3CA* present in SK were GC:AT transitions. The diversity of *FGFR3* nucleotide substitutions reported in these lesions points to the selection of variants because of their biological properties rather than to a pivotal role of a carcinogen fingerprint associated with UV exposure in the skin (32).

This is a comprehensive study of *PIK3CA* mutations in human skin and, more generally, of point mutations in an oncogene of the PI3K pathway in benign lesions without malignant potential. The PI3K pathway is thought to play a predominant role in cell

survival; mutations in genes coding for proteins acting upstream of PIKs, such as Ras genes or tyrosine kinase growth factor receptors, are thought to affect more directly cell proliferation. Pathologic activation of the FGFR3/PI3K/AKT pathway seems to be a frequent event in the pathogenesis of human EN and SK. That the majority of EN and SK in this study revealed a WT status in both *PIK3CA* and *FGFR3* suggests that additional genes of this or of other pathways contribute to the pathogenesis of acanthotic skin tumors.

In addition, we report previously undescribed oncogenic mutations in two different genes occurring concomitantly in a completely benign lesion, whether congenital or acquired. That the majority of SK appear to be clonal (33) lends support to the notion that both mutations may occur in the same cell, although more work is required to conclusively establish this fact. Oncogenic germ-line *FGFR3* mutations cause short limb dysplasia through the activation of Stat1 and the up-regulation of p21, thus leading to premature cell cycle exit in chondrocytes (34). We have proposed that this mechanism may, at least in part, be related to the association of *FGFR3* mutations with good prognosis in patients with superficial papillary bladder tumors (25). In addition, we have proposed that, in bladder tumors, *PIK3CA* mutations may bypass a putative growth disadvantage effect provided by *FGFR3* mutations (26). The findings in benign skin lesions reported here clearly indicate that, at least in keratinocytes, the presence of mutations in these two genes does not imply the malignant phenotype, supporting the notion that other genetic alterations, possibly in tumor suppressor genes, must occur to acquire a “progressor” phenotype. Furthermore, our findings suggest that the accumulation of mutations in oncogenes may effectively contribute to activate protective cellular mechanisms that act as a barrier for tumor development (35). Sarkisian *et al.* (36) have recently demonstrated the relevance of “the level of signaling” to the activation of senescence in a study using transgenic mice. In these mice, an oncogenic mutated Ras gene was expressed in the mammary gland under the control of a doxycycline-inducible promoter. Near-physiologic levels of oncogenic Ras led to hyperproliferation of epithelial cells, whereas high levels of Ras expression led to the activation of a senescence program. Importantly, high levels of oncogenic Ras failed to activate the senescence program in mice in which the *Ink4a-Arf* locus had been inactivated (36). Multiple mutations occurring in either a single oncogene, as is the case for *FGFR3* or *PIK3CA* in benign skin tumors, or in multiple oncogenes may provide sufficient signals to activate a senescence program in humans as well. Interestingly, inhibition of the PI3K pathway has recently been shown to be required for Ras-induced senescence *in vitro* (37).

Our findings also bear evidence to the ability of abnormal PI3K signaling in the activation of senescence. Until now, genes located mainly upstream of PI3K have been reported to activate senescence (i.e., receptor tyrosine kinases, *Ras*, *B-RAF*, and *NF1*) (38, 39). This observation is in agreement with the activation of senescence upon acute *Pten* inactivation in the mouse (40). Mallette *et al.* (41) have reported that the DNA damage response (DDR) is involved in the induction of senescence mediated by oncogenic Ras and Stat5, and it will be important to determine whether aberrant PI3K signaling is also able to activate the DDR (41).

Overall, our findings extend the spectrum of proliferative lesions harboring *PIK3CA* mutations and underline the relevance of understanding the mechanisms involved in early steps of tumor development in human carcinogenesis. The skin pro-

vides a potent model for such studies given its ready accessibility for visual, pathological, and genetic examination.

## Materials and Methods

**Patients and Samples.** The files of the Department of Pathology of Hospital del Mar and Hospital de Sant Pau, the Department of Dermatology of the University of Regensburg, and the Department of Dermatohistopathology, Friedrichshafen, were searched to retrieve diagnostic slides corresponding to cases labeled as “epidermal nevus” ( $n = 33$ ) and “seborrheic keratosis” ( $n = 62$ ). Cases diagnosed as nonorganoid nonepidermolytic EN showing the “common” histopathological features (acanthosis, papillomatosis, and a variable degree of hyperkeratosis), as well as cases showing an acrokeratosis verruciformis-like findings, or an SK-like pattern were included in the study. For SK, the three major histologic subtypes (acanthotic, hyperkeratotic, and adenoid) were retrieved. H&E-stained sections were reviewed by an experienced dermatopathologist, and cases fulfilling criteria for the appropriate diagnoses were selected for study. The study was approved by the Ethics Committees of all participating institutions and was performed according to the Declaration of Helsinki.

**PIK3CA and FGFR3 Mutation Analysis.** Microdissection, DNA extraction from formalin-fixed paraffin-embedded tissue, control samples, primers, and PCR conditions to amplify exons 9 and 20 of *PIK3CA* and exons 7, 10, and 15 of *FGFR3* have been reported (16, 26). DNA from unaffected tissue was used to determine the somatic nature of the variants.

PCRs were done by using 10–50 ng of DNA/0.2  $\mu\text{mol/liter}$  of each primer/200  $\mu\text{mol/liter}$  deoxynucleotide triphosphates/3.5 mmol/liter  $\text{MgCl}_2$ /1 $\times$  PCR II buffer/1.5 units of Amplitaq Gold DNA Polymerase (Applied Biosystems, Foster City, CA). PCR conditions were as follows: 94°C (10 min) for one cycle; 94°C (40 sec), 60°C (40 sec), and 72°C (40 sec) for 42 cycles; and a final extension step of 72°C (10 min). In all experiments, several reactions were included in the absence of DNA to rule out contamination by PCR products. All mutations were confirmed by analyzing the products of a second independent PCR as well as by sequencing of both strands of PCR products. In addition, *FGFR3* mutations for all SK in this study and 9 of 33 EN were analyzed by using a SNaPshot multiplex assay, as described (10, 42).

**Statistical Analyses.** Independence of *PIK3CA* and *FGFR3* mutational status was tested by applying  $\chi^2$  Mantel–Haenszel test. The trend of *PIK3CA* mutational prevalence according to sex, age, localization, and histologic subtype was estimated by using Fisher’s exact and Mann–Whitney tests.

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