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ORIGINAL REPORT

RAS Mutations Are Associated With the Development of Cutaneous Squamous Cell Tumors in Patients Treated With RAF Inhibitors

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A B S T R A C T

Purpose

RAF inhibitors are effective against melanomas with *BRAF* V600E mutations but may induce keratoacanthomas (KAs) and cutaneous squamous cell carcinomas (cSCCs). The potential of these agents to promote secondary malignancies is concerning. We analyzed cSCC and KA lesions for genetic mutations in an attempt to identify an underlying mechanism for their formation.

Methods

Four international centers contributed 237 KA or cSCC tumor samples from patients receiving an RAF inhibitor (either vemurafenib or sorafenib; n = 19) or immunosuppression therapy (n = 53) or tumors that developed spontaneously (n = 165). Each sample was profiled for 396 known somatic mutations across 33 cancer-related genes by using a mass spectrometric–based genotyping platform.

Results

Mutations were detected in 16% of tumors (38 of 237), with five tumors harboring two mutations. Mutations in *TP53*, *CDKN2A*, *HRAS*, *KRAS*, and *PIK3CA* were previously described in squamous cell tumors. Mutations in *MYC*, *FGFR3,* and *VHL* were identified for the first time. A higher frequency of activating *RAS* mutations was found in tumors from patients treated with an RAF inhibitor versus populations treated with a non–RAF inhibitor (21.1% *v* 3.2%; $P < .01$), although overall mutation rates between treatment groups were similar (RAF inhibitor, 21.1%; immunosuppression, 18.9%; and spontaneous, 17.6%; P = not significant). Tumor histology (KA *v* cSCC), tumor site (head and neck *v* other), patient age (≤ 70 *v* > 70 years), and sex had no significant impact on mutation rate or type.

Conclusion

Squamous cell tumors from patients treated with an RAF inhibitor have a distinct mutational profile that supports a mechanism of therapy-induced tumorigenesis in *RAS*-primed cells. Conceivably, cotargeting of MEK together with RAF may reduce or prevent formation of these tumors.

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INTRODUCTION

At least 50% of all melanomas carry an activating mutation in the *BRAF* oncogene.[1] In the advanced setting, the treatment of these melanomas with the selective RAF inhibitors vemurafenib (formerly PLX4032 or RG7204) and GSK2118436 has yielded response rates of 50% to 80%[2-4] and an improvement in overall survival when compared with conventional chemotherapy.[5] Similar to patients treated with other small-molecule kinase inhibitors, patients treated with a selective RAF inhibitor frequently experience skin toxicities.[6] However, a striking distinction of these agents has been the development of skin tumors in the form of keratoacanthomas (KAs) or cutaneous squamous cell carcinomas (cSCCs) in up to approximately 25% of patients.[2,4,5] These lesions most frequently develop within 8 to 12 weeks of beginning therapy. Similar treatment-related skin neoplasms have been described with the structurally unrelated multikinase inhibitor sorafenib.[7,8] Sorafenib has been reported to have pan-RAF inhibitory properties,[9] although the overall cellular potency of this compound

against RAF proteins is much less pronounced when compared with selective inhibitors.[10] Perhaps not surprisingly, sorafenib-induced skin tumors occur much less frequently and are more delayed in onset.[7,8] Together, these observations suggest that RAF inhibition may play a direct role in the development of skin tumors.

The concept that a targeted therapy that blocks an oncogenic pathway in one cell type may promote tumorigenesis in another is both novel and potentially concerning. Given that RAF inhibitors will likely gain widespread use in melanoma and perhaps other cancers, deciphering the molecular basis of inhibitor-induced cutaneous neoplasms is essential. One potential mechanism is suggested by recent preclinical experiments demonstrating that while RAF inhibitors inhibit mitogen-activated protein kinase (MAPK) signaling in *BRAF*-mutant cancer cells, they may also cause a paradoxical increase in MAPK signaling in the context of mutated or activated *RAS*. Toward this end, *RAS* mutations have previously been identified in actinic keratoses[11-13]—premalignant skin lesions with the potential to transform into cSCCs.[14] We therefore hypothesized that *RAS* activation in certain cutaneous cell subpopulations might interact with RAF inhibitor therapy to promote cell proliferation, ultimately resulting in KAs and cSCCs.

To test this hypothesis, we used a mass spectrometric genotyping platform (OncoMap) to generate mutational profiles for KA and cSCC lesions that developed in patients treated with an RAF inhibitor. As a comparator, we evaluated similar tumors that developed spontaneously or in the setting of immunosuppressive therapy.

METHODS

Tumor Specimens

Archival formalin-fixed paraffin-embedded (FFPE) cSCC and KA tumor specimens were collected from four international centers: the University of Essen, Essen, Germany; the Peter MacCallum Cancer Centre, East Melbourne, Australia; the University Hospital Zurich, Zurich, Switzerland; and the Gustave Roussy Institute, Villejuif, France. These samples were enriched for tumors that developed in patients undergoing treatment with an RAF inhibitor (vemurafenib or sorafenib) or immunosuppressive therapy for solid organ or bone marrow transplantations. Relevant clinical data were obtained from patients' medical records, and all samples were de-identified before analysis. The study was conducted with the approval of local institutional review boards.

DNA Preparation

Each tumor specimen was independently reviewed by two dermatopathologists to confirm the diagnosis. Tumor-rich areas (> 70% purity) were dissected and scraped from consecutive unstained FFPE slides. Genomic DNA was extracted by using the Qiagen DNeasy extraction kit (Qiagen, Valencia, CA) per the manufacturer's instructions. DNA quality was assessed by quantification with Picogreen and polymerase chain reaction amplification of fragments 100 to 200 base pairs (bp) long.

Mass Spectrometric Genotyping

High-throughput mutation profiling was performed on each sample by using the OncoMap platform. As previously described,[15] this approach interrogated 396 mutations across 33 known oncogenes and tumor suppressor genes. Genomic DNA obtained from tumor samples was initially screened by using iPLEX (Sequenom, San Diego, CA) genotyping, and candidate mutations were validated by using homogeneous mass extension chemistry on unamplified DNA. The cancer gene mutations interrogated by OncoMap were selected on the basis of a combination of historically documented mutation frequencies and their potential as therapeutic targets.[15,16] Primers and probes were designed by using Sequenom MassARRAY Assay Design 3.0 software (Sequenom), as described previously.[15,16]

Statistical Methods

For categorical comparisons, we performed either Fisher's exact test or the χ^2 test by using GraphPad Prism version 5.0 (GraphPad Software, La Jolla, CA). All tests were two-sided, and a threshold of $P < .05$ was used to define statistical significance.

RESULTS

Characteristics of Clinical Tumor Samples

A total of 237 FFPE clinical tumor specimens were evaluated for this study, consisting of 191 cSCCs and 46 KAs (Table 1). One hundred sixty-five samples were classified as "spontaneous" (ie, they originated in patients who did not receive either significant immunosuppressive therapy or small-molecule RAF inhibitor therapy); included were samples from four patients receiving cytotoxic chemotherapy, two patients taking thalidomide, one patient receiving interferon, and one patient who was HIV-positive. Patients undergoing significant immunosuppressive therapy contributed 53 samples (47 samples from solid organ transplantations, three from bone marrow transplantations, and one sample each from patients with severe psoriasis, aplastic anemia, and unknown), and 19 samples were derived from patients receiving small-molecule RAF inhibitors. Seven patients enrolled in phase I and II studies of vemurafenib contributed 10 lesions (four lesions from one patient), with lesions being excised between 48 and 107 days (mean, 70 days) after commencing vemurafenib. Nine samples were from patients receiving sorafenib for 3 to 9 months. Not unexpectedly, surrogate markers of higher ultraviolet radiation exposure (increased age, head and neck site) were statistically favored in the spontaneous versus nonspontaneous cohorts ($P < .01$), likely reflecting the lack of an additional precipitant when compared with the treatment groups.

Mutations in cSCC and KA Detected by Mass Spectrometric Genotyping

The OncoMap platform identified mutations in 38 of the 237 samples tested. Five samples exhibited co-occurring mutations; thus, a total of 43 mutations were detected (Fig 1; Table 2). The overall frequency of mutations was not significantly different between samples in the RAF inhibitor therapy, immunosuppression therapy, or spontaneous groups (21.1%, 18.9%, and 17.6%, respectively) nor did the frequency vary with patient age, sex, or tumor site. Furthermore, there was a similar rate and range of mutations between the cSCCs and KAs.

Mutations were detected across eight different cancer-related genes. In keeping with previous studies,[17]) the most frequently involved genes were *TP53* (n = 11), *CDKN2A* (n = 12), and the *RAS* isoforms *HRAS* (n = 10) and *KRAS* (n = 1). Not previously identified in cSCCs and KAs were mutations in *PIK3CA* (n = 5), *FGFR3* (n = 2), *MYC* (n = 1), and *VHL* (n = 1).

RAS Mutations Occur More Frequently in Tumors From Patients Treated With RAF Inhibitors

Tumors from the cohort of patients treated with an RAF inhibitor were enriched for *HRAS* mutations despite similar rates of total mutations between groups. Known activating mutations in *HRAS*

Table 1. Clinicopathologic Characteristics of Squamous Cell Tumor Samples

Characteristic	RAF-Inhibitor Treated (n = 19)		Immunosuppressed (n = 53)		Spontaneous (n = 165)		Total (N = 237)	
	No.	%	No.	%	No.	%	No.	%
Sex								
Male	10	52.6	41	77.4	113	68.5	164	69.2
Female	9	47.4	12	22.6	52	31.5	73	30.8
Age, years								
Range	44-89		42-83		26-104		26-104	
Mean	64.6		64.2		76.9		73.1	
SEM	2.6		1.3		0.9		0.8	
Site								
Head and neck	5	26.3	24	45.3	102	61.8	131	55.3
Trunk	2	10.5	3	5.7	5	3.0	10	4.2
Upper limb	7	36.8	13	24.5	24	14.5	44	18.6
Lower limb	5	26.3	10	18.9	25	15.2	40	16.9
Genital	—		—		5	3.0	5	2.1
Unknown	—		3	5.7	4	2.4	7	3.0
Histology								
cSCC	9	47.4	48	90.6	134	81.2	191	80.6
KA	10	52.6	5	9.4	31	18.8	46	19.4

Abbreviations: cSCC, cutaneous squamous cell carcinoma; KA, keratoacanthoma; SEM, standard error of the mean.

(Q61L, G12D) were identified in 30% (95% CI, 10% to 61%) of samples from patients treated with vemurafenib and 11% (95% CI, < 0.01% to 46%) of samples from patients treated with sorafenib. Combined, *HRAS* mutations were found in four of the 19 samples from patients treated with an RAF inhibitor compared with six of 218 *HRAS* mutations (21.1% *v* 2.8%; $P < .01$) and a single *KRAS* mutant (all *RAS* mutations, 21.1% *v* 3.12%; $P < .01$) in samples treated with a non-RAF inhibitor. No *NRAS* mutations were identified in this study. Furthermore, in the cohort of patients treated with an RAF inhibitor, no activating mutations were identified in 11 receptor tyrosine kinases that are commonly mutated in human cancers and that function upstream of *RAS* (*CSF1R*, *EGFR*, *ERBB2*, *FGFR1*, *FGFR2*, *FGFR3*, *FLT3*, *KIT*, *MET*, *PDGFRA*, and *RET*).

Surprisingly, we also identified *BRAF* V600E mutations in two samples from patients treated with vemurafenib for *BRAF* V600E-mutant metastatic melanoma (samples 3 and 39). Further immunohistochemical studies for AE1/AE3 (squamous cell carcinoma), S100, and melan-A (melanoma) identified clearly separate populations of malignant squamous cells and melanocytes in close proximity, with one sample showing evidence of metastatic melanoma cells within the lymphovascular space (Appendix Fig A1, online only). The *BRAF* mutations in these samples were therefore attributable to melanocytic contamination, and these mutations were not included in our statistical analysis.

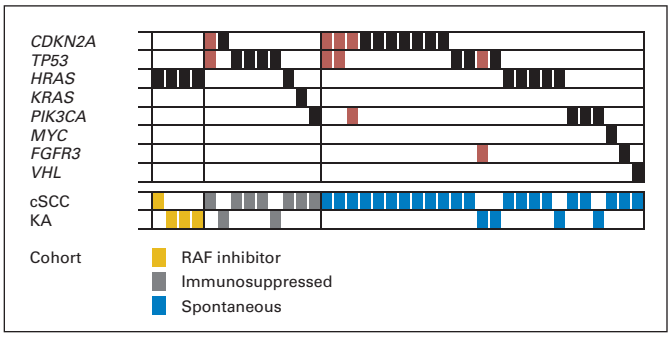

Fig 1. Mutually exclusive and co-occurring mutations in human squamous cell tumors. Each column describes an individual sample with a detected mutation. Affected genes are listed in rows, with single mutations indicated by black bars and co-occurring mutations indicated by red bars. Histology (cutaneous squamous cell carcinoma [cSCC] and keratoacanthoma [KA]) and treatment cohort (RAF-inhibitor, gold; immunosuppressed, gray; spontaneous, blue) are detailed on the bottom two rows.

DISCUSSION

Recent preclinical studies have identified the potential for selective RAF inhibitors to augment MAPK pathway activation in the context of activated *RAS*. In these systems, signaling occurred preferentially through C-RAF, with RAF inhibitors thought to induce a conformational change in C-RAF heterodimers or B-RAF homodimers that resulted in pathway hyperactivity.[18-21] In our study, we found that squamous cell tumors arising in patients treated with an RAF inhibitor are enriched for *RAS* mutations when compared with those from untreated patients. This finding suggests that the formation of these tumors is not due to a direct mutagenic event of RAF inhibitor therapy but is caused, at least in part, by a pro-proliferative interaction between RAF inhibitors and latent *RAS* mutant keratinocytes.

Unlike in other common skin cancers such as melanoma or basal cell carcinoma, a predominant mutational driver has yet to be identified in cSCC. *H-*, *K-*, and *NRAS* mutations occur infrequently in cSCC from the general[11,13,22,23] and immunosuppressed[24] populations and are reported at similarly low frequencies (1% to 12%) in premalignant actinic keratoses.[11-13,25] The presence of *RAS* mutations in these premalignant lesions (common in sun-damaged skin) suggests that ultraviolet radiation can induce *RAS* mutations in keratinocytes but that these mutations are not sufficient to induce malignant transformation. When *RAS*-activated keratinocytes are exposed to RAF inhibition, additional checks and balances may be exceeded, resulting in

Table 2. Characteristics of Samples With Detected Mutations

Patient	Sex	Age (years)	Histology	Site	Mutation Type	Patient Cohort	Treatment
1	Male	89	cSCC	Arm	*HRAS* Q61L	RAF kinase inhibitor	Vemurafanib
2	Female	54	KA	Back	*HRAS* Q61L	RAF kinase inhibitor	Vemurafanib
3	Male	71	KA	Chest	*HRAS* G12D; *BRAF* V600E	RAF kinase inhibitor	Vemurafanib
4	Male	53	KA	Thigh	*HRAS* Q61L	RAF kinase inhibitor	Sorafenib
5	Male	59	cSCC	Ear	*CDKN2A* P114L; *TP53* R248W	Immunosuppression	Methotrexate, cyclosporin, efalizumab, adalimumab
6	Male	82	KA	Lower arm	*CDKN2A* R80*	Immunosuppression	Cyclosporin
7	Male	67	cSCC	Lower leg	*TP53* R273H	Immunosuppression	Cyclosporin
8	Male	68	cSCC	Face	*TP53* R248Q	Immunosuppression	Drugs not specified
9	Male	74	cSCC	Leg	*TP53* R248W	Immunosuppression	Cyclosporin
10	Female	61	KA	N/A	*TP53* R248Q	Immunosuppression	Tacrolimus, mycophenylate mofitil, azathioprine
11	Male	60	cSCC	Lower arm	*HRAS* Q61K	Immunosuppression	Cyclosporin
12	Male	76	cSCC	Hand	*KRAS* G12D	Immunosuppression	Cyclosporin
13	Female	42	cSCC	Chest	*PIK3CA* E545K	Immunosuppression	Rapamycin, sirolimus
14	Male	80	cSCC	Hand	*CDKN2A* H83Y; *TP53* R248Q	Spontaneous	No treatment
15	Male	69	cSCC	Head	*CDKN2A* R58*; *TP53* R248W	Spontaneous	No treatment
16	Male	60	cSCC	Leg	*CDKN2A* R80*; *PIK3CA* E545K	Spontaneous	Methotrexate, cisplatin, fluorouracil, bleomycin
17	Male	81	cSCC	Face	*CDKN2A* P114L	Spontaneous	No treatment
18	Male	79	cSCC	Face	*CDKN2A* P114L	Spontaneous	No treatment
19	Male	72	cSCC	Scalp	*CDKN2A* R58*	Spontaneous	No treatment
20	Male	104	cSCC	Head	*CDKN2A* R58*	Spontaneous	No treatment
21	Male	71	cSCC	Head	*CDKN2A* H83Y	Spontaneous	No treatment
22	Male	77	cSCC	Ear	*CDKN2A* P114L	Spontaneous	No treatment
23	Male	65	cSCC	Hand	*CDKN2A* E88*	Spontaneous	No treatment
24	Male	81	cSCC	Lower arm	*TP53* R248W	Spontaneous	No treatment
25	Male	83	cSCC	Ear	*TP53* R248W	Spontaneous	No treatment
26	Female	88	KA	Cheek	*TP53* R248W; *FGFR3* G370C	Spontaneous	No treatment
27	Female	77	KA	Cheek	*TP53* R273C	Spontaneous	No treatment
28	Male	81	cSCC	Cheek	*HRAS* Q61L	Spontaneous	No treatment
29	Male	75	cSCC	Ear	*HRAS* Q61L	Spontaneous	No treatment
30	Female	77	cSCC	Genital	*HRAS* Q61L	Spontaneous	No treatment
31	Male	75	cSCC	Auricle	*HRAS* G12V	Spontaneous	No treatment
32	Female	26	KA	Lip	*HRAS* Q61L	Spontaneous	No treatment
33	Male	77	cSCC	Arm	*PIK3CA* E542K	Spontaneous	No treatment
34	Female	81	cSCC	Forehead	*PIK3CA* E545K	Spontaneous	No treatment
35	Female	62	KA	Lower Leg	*PIK3CA* H1047R	Spontaneous	No treatment
36	Female	78	cSCC	Upper arm	*MYC* A59V	Spontaneous	No treatment
37	Male	57	cSCC	Face	*FGFR3* G370C	Spontaneous	No treatment
38	Male	52	cSCC	Lower leg	*VHL* P81S	Spontaneous	No treatment
39	Male	61	cSCC	Arm	*BRAF* V600E	RAF kinase inhibitor	Vemurafanib

Abbreviations: cSCC, cutaneous squamous cell carcinoma; KA, keratoacanthoma; NA, not available.
*Denotes stop codon.

abnormal cell growth and, ultimately, progression to either KA or cSCC. This model would fit the clinical observations that the KA and cSCC lesions most commonly develop in the first 8 to 12 weeks after initiating RAF inhibitor therapy and are more frequent in patients with increased sun exposure, supporting the existing presence of a predisposing lesion. In some cases, these tumors have spontaneously regressed on treatment discontinuation. The mechanisms for this regression are not clear but could involve altered signaling, activation of a senescence-like program, or induction of an immune response to the KA and cSCC lesions. Regarding the latter, some studies have noted the presence of cytotoxic T lymphocytes, Langerhans cells, and $CD30^+$ cells within KA lesions.[26-29]

So far, the cutaneous squamous cell tumors that have developed in patients receiving vemurafenib and GSK2118436 have all been well differentiated and have not metastasized or recurred after complete excision.[4,30] Sorafenib is a multikinase inhibitor whose effects against RAF isoforms are considerably less pronounced in vivo than those of vemurafenib and GSK2118436, which may explain the lower incidence of skin tumors seen with this drug.[7,9] Notably, sorafenib is ineffective against *BRAF*-mutant melanoma; this reduced in vivo potency may relate in part to its higher affinity for the inactive conformation of this kinase than the active form acquired with a *BRAF* V600E mutation.[31] In the context of patients with advanced melanoma and its associated poor prognosis, development of KA or cSCC as a treatment-related adverse effect has been considered acceptable to both patients and clinicians. However, the confirmation of a clinically significant interaction between selective RAF inhibitors and *RAS*-activated cells raises several additional considerations.

The most concerning of these is the potential for secondary malignancies other than those of the skin. *RAS* mutations are estimated to occur in 30% of all human cancers, with a significant further proportion having either activating mutations or overexpression of upstream receptor tyrosine kinases (such as HER2, c-KIT, and EGFR[32]). Furthermore, apart from occurring in actinic keratoses, *RAS* mutations also occur as early genetic events in a range of premalignant lesions. For instance, *KRAS* mutations represent early genetic events in colon carcinogenesis: they are present in up to 50% of colonic adenomas.[33,34] They are also found at increasing frequency with progressively higher grades of pancreatic intraepithelial neoplasia, correlating with increasing risk of progressing to carcinoma[35] and are more commonly detected in the bronchial washings of smokers compared with those of nonsmokers.[36] In principle, exposure of these cell subpopulations to RAF inhibitors could promote clonal expansion and propel them toward permanent malignant transformation.

Why have no such extracutaneous tumors been detected thus far? One possible reason might be that the median treatment duration of vemurafenib and GSK2118436 (6 to 9 months) has not been long enough for these lesions to manifest. Alternatively, some such events may have been misinterpreted as melanoma progression. It is also possible that *RAS*-mutant cell populations in other organs may not undergo the sustained proliferation typical of fully malignant neoplasms. Concerns of possible tumorigenic complications may become heightened by the inevitable transition of these agents into the adjuvant setting, in which treatment duration could last 1 to 2 years, and the clinical impact of any secondary malignancies might be increased. Thus, for patients participating in the initial adjuvant studies of these agents, careful surveillance will be essential. Aggressive histologic characterization (which may include tumor mutational profiling) may be needed for new lesions that arise during adjuvant treatment using selective RAF inhibitors.

Our findings also illustrate how proposed strategies to overcome resistance and potential strategies to prevent secondary tumor development may converge going forward. To date, several mechanisms of secondary RAF inhibitor resistance have been postulated. These include acquired mutations in *NRAS* and overexpression of *PDGFR-β* and *IGFR*, both of which can operate upstream of *RAS*.[37,38] These models may suggest a switch to C-RAF–driven MAPK signaling[39] that is operant in some cases. Conceivably, such resistance mechanisms might be circumvented through the development of more potent RAF inhibitors that abrogate the mechanism of RAF activation observed with existing compounds, or alternatively by blocking signaling downstream of both B-RAF and C-RAF by targeting MEK or ERK. Clinical studies of concomitant RAF and MEK inhibitors have commenced in an attempt to prolong the effectiveness of (or overcome resistance to) RAF blockade in *BRAF* V600E-mutant melanomas. Preliminary results suggest that this combination may reduce the incidence of RAF inhibitor–induced KA and cSCC lesions.[40] We speculate that such combinations may also suppress proliferation of *RAS*-activated nonmelanoma cell populations elsewhere in the body.

More generally, our study provides one of the largest mutational studies of cSCCs and KAs reported to date. Consistent with the previous literature, the most frequently mutated genes were *TP53* and *CDKN2A*.[41,42] However, we detected substantially lower rates of mutations in these genes (4.6% and 5.1%, respectively) compared with those previously reported (44.4% and 24.5%, respectively), most likely reflecting the known limitation of genotyping-based platforms in detecting mutations in tumor suppressor genes. Inactivating mutations are more diverse and therefore harder to cover with multiplexed, mutation-specific assays when compared with the limited number of functional activating mutations in oncogenes. For instance, our assays covered only 11% of *TP53* and 24% of *CDKN2A* mutations previously identified in cSCC, whereas 100% of previously described *H-*, *K-*, and *N-RAS* mutations were assessed. Of note, *PTCH1* was reportedly mutated in more than 5% of cSCC samples; however, this finding was limited to only one study,[43] in which all three nonsilent mutations occurred in patients with a history of multiple basal cell carcinomas (*PTCH1* was not assessed in our study). We identified novel mutations in four genes: *PIK3CA*, *FGFR3*, *MYC*, and *VHL*, but these occurred in no more than 2% of samples. No difference was identified in the rate or types of mutations between cSCCs and KAs. The histologic and biologic distinction between these entities remains an area of controversy for dermatopathologists.[44,45]

Finally, although we have identified mutations in *RAS* in roughly 20% of squamous cell tumors that developed during therapy with an RAF inhibitor, tumorigenic mechanisms operant in *RAS*-negative KA and cSCC lesions remain unclear. It is possible that the frequency of *RAS* mutations in treatment-induced KAs and cSCCs will rise as larger patient cohorts treated at maximal RAF inhibitor doses are analyzed. Additional mechanisms may involve activation of upstream effectors (eg, receptor tyrosine kinases) by gene amplification or overexpression, which were not examined here. The application of more comprehensive mutation profiling technologies such as targeted, massively parallel sequencing may shed additional light on the full spectrum of genomic alterations that drive the biology of these squamous cell tumors. Several other mechanisms have been proposed by which tumors may develop in the presence of sorafenib, with most being related to its multikinase activity.[46,47] How such mechanisms might relate to those linked to selective RAF inhibitors remains unclear.

In summary, exposure to selective RAF inhibitors may lead to pro-proliferative effects on *RAS*-primed cells. This has already manifested clinically in the form of squamous cell tumors, but the potential may also exist to promote growth of other extracutaneous neoplasms (and to promote resistance in melanoma) by the same mechanism. Cotargeting of MEK (or, in the future, ERK) together with RAF may block this effect. Thus, compound MAP kinase pathway inhibition may simultaneously enhance antitumor efficacy and restrict proneoplastic adverse effects of single-agent RAF inhibition.

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REFERENCES

1. Platz A, Egyhazi S, Ringborg U, et al: Human cutaneous melanoma: A review of NRAS and BRAF mutation frequencies in relation to histogenetic subclass and body site. Mol Oncol 1:395-405, 2008

2. Flaherty KT, Puzanov I, Kim KB, et al: Inhibition of mutated, activated BRAF in metastatic melanoma. N Engl J Med 363:809-819, 2010

3. Kefford R, Arkenau H, Brown MP, et al: Phase I/II study of GSK2118436, a selective inhibitor of oncogenic mutant BRAF kinase, in patients with metastatic melanoma and other solid tumors. J Clin Oncol 28:611s, 2010 (suppl; abst 8503)

4. Ribas A, Kim KB, Schuchter LM, et al: BRIM-2: An open-label, multicenter phase II study of vemurafenib in previously treated patients with BRAF V600E mutation-positive metastatic melanoma. J Clin Oncol 29:528s, 2011 (suppl; abst 8509)

5. Chapman PB, Hauschild A, Robert C, et al: Improved survival with vemurafenib in melanoma with BRAF V600E mutation. N Engl J Med 364:2507-2516, 2011

6. Robert C, Soria JC, Spatz A, et al: Cutaneous side-effects of kinase inhibitors and blocking antibodies. Lancet Oncol 6:491-500, 2005

7. Arnault JP, Wechsler J, Escudier B, et al: Keratoacanthomas and squamous cell carcinomas in patients receiving sorafenib. J Clin Oncol 27:e59-e61, 2009

8. Dubauskas Z, Kunishige J, Prieto VG, et al: Cutaneous squamous cell carcinoma and inflammation of actinic keratoses associated with sorafenib. Clin Genitourin Cancer 7:20-23, 2009

9. Wilhelm SM, Carter C, Tang L, et al: BAY 43-9006 exhibits broad spectrum oral antitumor activity and targets the RAF/MEK/ERK pathway and receptor tyrosine kinases involved in tumor progression and angiogenesis. Cancer Res 64:7099-7109, 2004

10. Whittaker S, Kirk R, Hayward R, et al: Gatekeeper mutations mediate resistance to BRAF-targeted therapies. Sci Transl Med 2:35ra41, 2010

11. Zaravinos A, Kanellou P, Spandidos DA: Viral DNA detection and RAS mutations in actinic keratosis and nonmelanoma skin cancers. Br J Dermatol 162:325-331, 2010

12. Nindl I, Gottschling M, Krawtchenko N, et al: Low prevalence of p53, p16(INK4a) and Ha-ras tumour-specific mutations in low-graded actinic keratosis. Br J Dermatol 156:34-39, 2007 (suppl 3)

13. Spencer JM, Kahn SM, Jiang W, et al: Activated ras genes occur in human actinic keratoses, premalignant precursors to squamous cell carcinomas. Arch Dermatol 131:796-800, 1995

14. Ko CJ: Actinic keratosis: Facts and controversies. Clin Dermatol 28:249-253, 2010

15. MacConaill LE, Campbell CD, Kehoe SM, et al: Profiling critical cancer gene mutations in clinical tumor samples. PLoS One 4:e7887, 2009

16. Thomas RK, Baker AC, Debiasi RM, et al: High-throughput oncogene mutation profiling in human cancer. Nat Genet 39:347-351, 2007

17. Wellcome Trust Sanger Institute: COSMIC database. http://www.sanger.ac.uk/genetics/CGP/cosmic/

18. Hatzivassiliou G, Song K, Yen I, et al: RAF inhibitors prime wild-type RAF to activate the MAPK pathway and enhance growth. Nature 464:431-435, 2010

19. Heidorn SJ, Milagre C, Whittaker S, et al: Kinase-dead BRAF and oncogenic RAS cooperate to drive tumor progression through CRAF. Cell 140:209-221, 2010

20. Poulikakos PI, Zhang C, Bollag G, et al: RAF inhibitors transactivate RAF dimers and ERK signalling in cells with wild-type BRAF. Nature 464:427-430, 2010

21. Halaban R, Zhang W, Bacchiocchi A, et al: PLX4032, a selective BRAF(V600E) kinase inhibitor, activates the ERK pathway and enhances cell migration and proliferation of BRAF melanoma cells. Pigment Cell Melanoma Res 23:190-200, 2010

22. Campbell C, Quinn AG, Rees JL: Codon 12 Harvey-ras mutations are rare events in non-melanoma human skin cancer. Br J Dermatol 128:111-114, 1993

23. Corominas M, Kamino H, Leon J, et al: Oncogene activation in human benign tumors of the skin (keratoacanthomas): Is HRAS involved in differentiation as well as proliferation? Proc Natl Acad Sci U S A 86:6372-6376, 1989

24. Pélisson I, Chardonnet Y, Euvrard S, et al: Low incidence of c-Ha-ras gene mutations in benign and malignant cutaneous lesions from transplant recipients. Int J Cancer 55:915-920, 1993

25. Taguchi M, Tsuchida T, Ikeda S, et al: Alterations of p53 gene and Ha-ras gene are independent events in solar keratosis and squamous cell carcinoma. Pathol Int 48:689-694, 1998

26. Batinac T, Zamolo G, Hadzisejdic I, et al: A comparative study of granzyme B expression in keratoacanthoma and squamous cell carcinoma. J Dermatol Sci 44:109-112, 2006

27. Bayer-Garner IB, Ivan D, Schwartz MR, et al: The immunopathology of regression in benign lichenoid keratosis, keratoacanthoma and halo nevus. Clin Med Res 2:89-97, 2004

28. Fernandez-Flores A: CD30+ cells in regressing keratoacanthoma and in non-keratoacanthomatous squamous cell carcinoma. Bratisl Lek Listy 109:508-512, 2008

29. Korenberg R, Penneys NS, Kowalczyk A, et al: Quantitation of S100 protein-positive cells in inflamed and non-inflamed keratoacanthoma and squamous cell carcinoma. J Cutan Pathol 15:104-108, 1988

30. Lacouture ME, McArthur GA, Chapman PB, et al: PLX4032 (RG7204), a selective mutant RAF inhibitor: Clinical and histologic characteristics of therapy-associated cutaneous neoplasms in a phase I trial. J Clin Oncol 28:634s, 2010 (suppl; abstr 8592)

31. Hauschild A, Agarwala SS, Trefzer U, et al: Results of a phase III, randomized, placebo-controlled study of sorafenib in combination with carboplatin and paclitaxel as second-line treatment in patients with unresectable stage III or stage IV melanoma. J Clin Oncol 27:2823-2830, 2009

32. Bos JL: ras oncogenes in human cancer: A review. Cancer Res 49:4682-4689, 1989

33. Fearon ER, Vogelstein B: A genetic model for colorectal tumorigenesis. Cell 61:759-767, 1990

34. Vogelstein B, Fearon ER, Hamilton SR, et al: Genetic alterations during colorectal-tumor development. N Engl J Med 319:525-532, 1988

35. Löhr M, Klöppel G, Maisonneuve P, et al: Frequency of K-ras mutations in pancreatic intraductal neoplasias associated with pancreatic ductal adenocarcinoma and chronic pancreatitis: A meta-analysis. Neoplasia 7:17-23, 2005

36. Yakubovskaya MS, Spiegelman V, Luo FC, et al: High frequency of K-ras mutations in normal appearing lung tissues and sputum of patients with lung cancer. Int J Cancer 63:810-814, 1995

37. Nazarian R, Shi H, Wang Q, et al: Melanomas acquire resistance to B-RAF(V600E) inhibition by RTK or N-RAS upregulation. Nature 468:973-977, 2010

38. Villanueva J, Vultur A, Lee JT, et al: Acquired resistance to BRAF inhibitors mediated by a RAF kinase switch in melanoma can be overcome by cotargeting MEK and IGF-1R/PI3K. Cancer Cell 18:683-695, 2010

39. Johannessen CM, Boehm JS, Kim SY, et al: COT drives resistance to RAF inhibition through MAP kinase pathway reactivation. Nature 468:968-972, 2010

40. Infante JR, Falchook GS, Lawrence DP, et al: Phase I/II study to assess safety, pharmacokinetics, and efficacy of the oral MEK 1/2 inhibitor GSK1120212 (GSK212) dosed in combination with the oral BRAF inhibitor GSK2118436 (GSK436). J Clin Oncol 29:526s, 2011 (suppl; abst CRA8503)

41. Boukamp P: Non-melanoma skin cancer: What drives tumor development and progression? Carcinogenesis 26:1657-1667, 2005

42. Pacifico A, Goldberg LH, Peris K, et al: Loss of CDKN2A and p14ARF expression occurs frequently in human nonmelanoma skin cancers. Br J Dermatol 158: 291-297, 2008

43. Ping XL, Ratner D, Zhang H, et al: PTCH mutations in squamous cell carcinoma of the skin. J Invest Dermatol 116:614-616, 2001

44. Robert C, Arnault JP, Mateus C: RAF inhibition and induction of cutaneous squamous cell carcinoma. Curr Opin Oncol 23:177-182, 2011

45. Ko CJ: Keratoacanthoma: Facts and controversies. Clin Dermatol 28:254-261, 2010

46. Williams VL, Cohen PR, Stewart DJ: Sorafenib-induced premalignant and malignant skin lesions. Int J Dermatol 50:396-402, 2011

47. Kwon EJ, Kish LS, Jaworsky C: The histologic spectrum of epithelial neoplasms induced by sorafenib. J Am Acad Dermatol 61:522-527, 2009