Vemurafenib Cooperates with HPV to Promote Initiation of Cutaneous Tumors

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Abstract

Treatment with RAF inhibitors such as vemurafenib causes the development of cutaneous squamous cell carcinomas (cSCC) or keratoacanthomas as a side effect in 18% to 30% of patients. It is known that RAF inhibitors activate the mitogen—activated protein kinase (MAPK) pathway and stimulate growth of RAS-mutated cells, possibly accounting for up to 60% of cSCC or keratoacanthoma lesions with RAS mutations, but other contributing events are obscure. To identify such events, we evaluated tumors from patients treated with vemurafenib for the presence of human papilloma virus (HPV) DNA and identified 13% to be positive. Using a transgenic murine model of HPV-driven cSCC (K14-HPV16 mice), we conducted a functional test to determine whether administration of RAF inhibitors could promote cSCC in HPV-infected tissues. Vemurafenib treatment elevated MAPK markers and increased cSCC incidence from 22% to 70% in this model. Furthermore, 55% of the cSCCs arising in vemurafenib-treated mice exhibited a wild-type Ras genotype, consistent with the frequency observed in human patients. Our results argue that HPV cooperates with vemurafenib to promote tumorigenesis, in either the presence or absence of RAS mutations. Cancer Res; 74(8); 1–8. ©2014 AACR.

Introduction

Nearly 50% of melanomas harbor a single activating Valine to Glutamic acid point mutation of the BRAF kinase (1). Constitutive BRAF activation leads to phosphorylation of the downstream effectors MAP–ERK kinase (MEK), then extracellular signal—regulated kinase (ERK), which in turn activates transcription factors that promote proliferation of cancer cells. Phase II and phase III clinical trial results in BRAFV600E-mutated melanoma patients treated with the RAF inhibitor, vemurafenib, demonstrated improved survival and dramatic reduction in tumor burden (2, 3). Among the most common side effects is the emergence of nonmelanocytic cutaneous tumors, including cutaneous squamous cell carcinomas (cSCC), keratoacanthomas, and verrucous papillomas (2–5). Preclinical data demonstrate that RAF inhibitors paradoxically stimulate ERK phosphorylation in RAS-mutated cancer cells (6–9) through allosteric and catalytic mechanisms that relieve the auto-inhibition of wild-type RAF kinase (10). It is therefore hypothesized that paradoxical activation of the mitogen—activated protein kinase (MAPK) pathway induces cSCC and keratoacanthomas tumorigenesis in patients treated with RAF inhibitors.

Several groups have described the genetics of keratoacanthomas and cSCC induced by RAF inhibitors. Most notably 18% to 60% of the clinical biopsies harbor a RAS mutation (11–13), and treatment with a vemurafenib analogue (PLX-4720) also decreased tumor latency in a mouse model for Hras-driven cSCC (13). However, 40% to 82% of keratoacanthomas and cSCCs from RAF inhibitor-treated patients are RAS wild-type, suggesting that accelerated oncogenesis of RAS-mutated cells is not the only mechanism involved. Among the other mutated genes identified in cutaneous lesions from RAF inhibitor-treated patients are FGFR3, TGFBR1, MYC, PIK3CA, and tumor suppressors TP53, CDKN2A, and VHL (4, 11). Although FGFR3 and TGFBR1 mutations may lead to RAS activation and therefore hyperactivate the MAPK pathway in response to RAF inhibitors, it is unclear how critical RAS-activating oncogenes are to vemurafenib-induced cSCC. Furthermore, the role of other drivers, such as infectious agents like human papilloma virus (HPV), has not been carefully examined. Therefore, we used a transgenic mouse model that expresses the HPV16 early virus (HPV), has not been carefully examined. Therefore, we used a transgenic mouse model that expresses the HPV16 early

Materials and Methods

HPV screening of clinical biopsies

Cellular DNA was extracted from biopsies of cutaneous lesions or normal skin. HPV types were identified using
RAS genotyping of clinical biopsies

Genomic DNA was extracted from 10 to 20-μm thick paraffin-embedded unstained slides of each skin lesion and full coding sequences of *HRAS* (NM_005343.2) exons 2 and 3, *KRAS* (NM_033360.2) and exons 2 and 3, *NRAS* (NM_002524.3). Sequences were analyzed by Sanger direct sequencing performed after PCR amplification of target exons. Sequencing reactions were carried out using the BigDye Terminator Cycle Sequencing Kit software according to the manufacturer’s recommendations (Applied Biosystems). Sequencing reactions were analyzed on a 48-capillary 3730 DNA Analyzer. Sequence reading and alignment were performed with Seqscape software (Applied Biosystems).

Tissue culture

Proliferation of A375, SW620 (from ATCC), and GTL-16 cells (from S. Giordano, Cancer Research and Treatment, Turin, Italy) was measured by Cell Titer-Glo (Promega) and Rat-1 cells (from J. Gorski, University of Wisconsin, Madison, WI) were grown in soft agar suspension. *p*ERK quantification by sandwich immunoassay (MSD). All cell lines were routinely inspected for morphologic changes and tested mycoplasma negative. A375, SW620, and GTL-16 were also inspected for DNA copy number and gene expression changes as previously described (17). Primary antibodies used for immunoblot analyses: MEK, ERK, pMEK, pERK (Cell Signaling Technology, 9122, 9102, 9121, and 9101, respectively).

Mice

FVB.Cg-Tg (KRT-14-HPV16) mice were obtained from Jackson Laboratories. Unless otherwise stated, mice 6 months old that had not yet developed cSCC received 15, 30, or 60 mg/kg once daily for 60 days of vemurafenib by oral gavage. For inhibitor combination studies, mice were given 3 mg/kg PD0325901 administered twice daily in addition to 60 mg/kg vemurafenib daily. Because cSCC most frequently occur on the ears or chest in this model, these tissues were collected from every mouse (in addition to any tumor present, regardless of location) and frozen in liquid nitrogen for PCR analysis or formalin fixed and paraffin embedded for histology. Nontransgenic littermates were used as negative controls. *Dusp6* mRNA expression levels were quantified by quantitative PCR (qPCR) from RNA in the collected tissues (Qiagen fibrous tissue RNA collection kit; Ambion cDNA synthesis kit). *Dusp6* mRNA was normalized to 18s rRNA (Applied Biosystems: Mm00518185_m1 and 4319413E).

Mouse histology

Tissues were formalin fixed and paraffin embedded. Sections were stained with hematoxylin and eosin or anti-pERK antibody (Cell Signaling Technology 9121). Slides were randomized and blinded before histology scores determined by a pathologist. Skin and tumor samples were ranked as normal, premalignant, or malignant cSCC (Fig. 3B). Premalignant histology included moderate to severe hyperplasia, dysplasia, and hyperkeratosis. K14-HPV16 cSCCs are marked by keratin “pearls” of invasive well-differentiated squamous carcinoma (WDSC) occurring primarily on the ventral surface of the ear, or by a keratin cap containing heterogeneous regions of moderate to poorly differentiated squamous carcinoma (M/PDSC) occurring primarily on the chest and trunk. Malignant phenotypes included any M/PDSC, WDSC, and keratoacanthoma-like lesions.

Vemurafenib paradoxically activates the MAPK pathway in RAF wild-type epidermis

To determine whether clinically relevant exposures to vemurafenib are sufficient to activate the MAPK pathway and promote cSCC tumorigenesis in vivo, nontransgenic FVB/N mice were treated with 60 mg/kg vemurafenib (equivalent to the phase III dose; ref. 2) for 60 days. Although none of the mice treated developed spontaneous tumors, subtle evidence of elevated MAPK activity was observed in epithelial tissues. A modest increase in nuclear phosphorylated ERK staining was observed in nonglandular gastric epithelium (Supplementary Fig. S2) and epidermis (Fig. 1A). Because transcriptional targets of the MAPK pathway are often a more sensitive marker for ERK activity (21), *Dusp6* mRNA levels were measured by qPCR and were found to be increased 3-fold in skin from vemurafenib-treated mice (Fig. 1B), demonstrating that vemurafenib paradoxically activates the MAPK pathway in genetically wild-type mouse epidermis, but is insufficient for cSCC tumorigenesis.

Vemurafenib paradoxically activates the MAPK pathway in RAF/RAS wild-type cells

Having observed elevated MAPK markers in vemurafenib-treated mice, we wished to test whether a RAS oncogene is
required for vemurafenib to promote tumorigenesis. To test this in vitro, three cancer cell lines with different RAS/RAF mutations were treated with vemurafenib. Although vemurafenib effectively inhibited ERK phosphorylation and proliferation of BRAF-mutated cells, ERK phosphorylation was stimulated and proliferation increased in RAS/RAF wild-type cells, though to markedly higher levels in the KRAS-mutated cell (Fig. 1C). High doses of vemurafenib similarly increased ERK phosphorylation and induced anchorage-independent growth of RAS/RAF wild-type Rat-1 fibroblasts (Fig. 1D), demonstrating that vemurafenib treatment is sufficient to activate the MAPK pathway and promote a transformation phenotype. However, the magnitude of activation may be dependent upon the underlying genetics, and vemurafenib-induced activation seems to be particularly exaggerated in RAS-mutated cells.

A subset of cutaneous lesions from vemurafenib-treated patients are HPV-positive and RAS wild-type

Given these observations, we hypothesized that paradoxical activation of the MAPK pathway by RAF inhibitor treatment may promote cSCC tumorigenesis only in the presence of preexisting genetic lesions. Several common tumor suppressor mutations have been identified in cSCCs from patients treated with RAF inhibitors, including TP53, CDKN2A, and VHL (4, 11). In addition to host mutations, the contribution of viral onco-genes has also been implicated in cSCC tumorigenesis. The majority of nonmelanocytic lesions occurring in vemurafenib-treated patients include several benign skin lesions with strong associations with HPV, including venereal warts and verrucous papillomas (5), which strongly implicates a role for HPV. Although the frequency is low, expression of HPV capsid protein has been observed in one cSCC from a patient treated with the RAF inhibitor dabrafenib (22), and HPV DNA was observed in a separate case study (23).

To determine whether an HPV infection may contribute to cutaneous tumor initiation in patients treated with vemurafenib, 62 benign or malignant cutaneous lesions from 44 patients were collected and tested for the presence of HPV DNA. Eight of the lesions (13%) tested positive for one of six HPV types (Fig. 2), indicating that HPV may contribute in a subset of cases. Because HPV can also be found on normal skin and may be considered commensal with human tissue, we tested several tumors from the same patients, including normal skin samples when possible. In four cases, patients with
HPV-positive tumors also had additional skin tumors or normal skin samples that were HPV negative. In one patient, two different HPV types (HPV9 and 49) were found in two distinct skin tumors, whereas three additional tumors from the same patient were negative for HPV DNA. Additional indirect evidence of a viral contribution was observed in several HPV-negative tumors. Koilocyte-like cells were observed in papillated epidermal hyperplasia with a thickening of the granular layer, as commonly seen in viral warts (Supplementary Fig. S1).

Because RAS mutations frequently occur in vemurafenib-induced patient tumors, each tumor was also genotyped for HRAS, KRAS, and NRAS mutations. As shown in Fig. 2A, 10 (16%) RAS mutations (5 HRAS and 5 KRAS mutations) were identified, which is approximately equivalent to the frequency of HPV-positive tissues. Although HPV DNA did not seem to associate with any histologic category, HRAS mutations were only observed in keratoacanthomas and KRAS mutations were exclusive to verrucous papillomas. Interestingly, co-occurrence of a RAS mutation and HPV was only observed for one HRAS-mutated keratoacanthoma (Fig. 2B), suggesting that if HPV infection contributes to tumor initiation in vemurafenib-treated patients, it primarily occurs independent of RAS mutations.

Vemurafenib promotes Ras wild-type tumorigenesis in K14-HPV16 mice

To test whether vemurafenib can promote cSCC tumorigenesis in HPV-infected keratinocytes, we used a transgenic mouse model that expresses the HPV16 early genes from the keratin 14 promoter (K14-HPV16; ref. 14). These mice exhibit epidermal hyperplasia, hyperkeratosis, and many develop cSCCs and keratoacanthoma-like tumors. To our knowledge, no mutations have been described in spontaneous K14-HPV16 cSCCs. Tumor-free mice between 5 and 7 months old were treated daily with vemurafenib and tumor volume was monitored for 60 days. Activation of the MAPK pathway in epidermis was both time and dose dependent. Low doses (15 and 30 mg/kg) of vemurafenib moderately increased dermal Dusp6 expression only at early time points, whereas 60 mg/kg showed sustained increase in Dusp6 expression at 12 and even 24 hours after treatment (Fig. 3A). Neither the 15 nor 30 mg/kg cohort significantly changed the frequency of cSCC and/or premalignant phenotypes. However, the frequency of cSCC was significantly increased from 22% in vehicle-treated mice to 70% in the 60 mg/kg vemurafenib cohort (Fig. 3B and C).

To test for the presence of Ras oncogenes, cSCCs were collected from both vemurafenib-treated and untreated K14-HPV16 mice and genomic exons were sequenced. Somewhat surprisingly, 100% (9/9) of vemurafenib naïve tumors sequenced carried an activating Ras mutation, eight of which were Kras mutated, and 1 Hras (Q61L) mutated (Fig. 3D), suggesting that a Ras oncogene is required for cSCC in this model. However, only 44% (4/9) of the cSCCs from vemurafenib-treated mice were Ras mutated (Fig. 3D), suggesting that the increase in cSCC frequency is due to an increase of Ras wild-type tumors. Tumors from both vehicle- and vemurafenib-treated mice were also stained for ERK phosphorylation, and areas of every tumor stained positive (Supplementary Fig. S3), suggesting that K14-HPV16 cSCC requires ERK activation. However, no other Ras activating mutations and no other known oncogenes were observed in the vemurafenib-treated, Ras wild-type tumors. Nor did any set of mutations distinguish Ras wild-type cSCC from Ras-mutated or vehicle-treated cSCC.

K14-HPV16 cSCC is MEK dependent

These sequencing data suggest that the requirement for a Ras mutation in K14-HPV16 cSCC is bypassed by vemurafenib treatment. Conceivably, cSCC in this model requires the MAPK pathway activation, which occurs frequently through a Ras mutation, but can also occur through paradoxical activation of ERK signaling in vemurafenib-treated mice. If correct, then blockade of the MAPK pathway downstream of RAF should prevent the increased incidence of cSCC caused by vemurafenib. Consistent with this hypothesis, cotreatment of a RAF and MEK inhibitor in clinical trials reduced the frequency of squamous cancers compared with RAF inhibitor treatment alone (24). To test whether this is also true for K14-HPV16 cSCC, mice were treated with a combination of vemurafenib and a MEK inhibitor, PD0325901. Dusp6 mRNA expression was elevated in vemurafenib-treated skin, and reduced to background levels by PD0325901 (Fig. 4A), showing that 3 mg/kg PD0325901 is sufficient to prevent any paradoxical
Vemurafenib Cooperates with HPV to Promote Tumorigenesis

MAPK activation caused by vemurafenib. Mice treated with PD0325901 also had reduced hyperkeratosis and improved grooming (Supplementary Fig. S4). Concomitant treatment of 60 mg/kg vemurafenib and 3 mg/kg PD0325901 also significantly reduced the frequency of cSCC from 70% in vemurafenib-treated mice to 17% in vemurafenib + PD0325901-treated mice (Fig. 4B). However, none of the mice treated with PD0325901 as a single agent developed cSCCs during the study, suggesting that MEK inhibition prevents cSCC tumor initiation in K14-HPV16 mice.

To test whether MEK activity is required for tumor maintenance, mice with established spontaneous cSCC were treated with PD0325901 as a single agent developed cSCCs during the study, suggesting that MEK inhibition prevents cSCC tumor initiation in K14-HPV16 mice.

Discussion

Although it is known that RAF inhibitors accelerate RAS-mutant tumor growth, it is unclear why roughly 40% to 80% of cSCCs from patients treated with vemurafenib are RAS wild-type (11, 13). In the present study, vemurafenib not only increased the incidence and accelerated tumorigenesis, but also clearly induced Ras wild-type cSCC in K14-HPV16 mice (Fig. 3C and D). This suggests that vemurafenib is required for initiation of cSCC in Ras wild-type cells, and may then explain the emergence of Ras wild-type cSCC in RAF inhibitor-treated patients. Also consistent with the clinical observations (24), cotreatment with the MEK inhibitor PD0325901 prevented vemurafenib-induced cSCC (Fig. 4B). Moreover, treatment of established cSCC with PD0325901 caused tumor regression (Fig. 5), demonstrating that MAPK activity is not only required for initiation, but also for cSCC maintenance. However, it is important to note that vemurafenib-induced cSCC was only observed in K14-HPV16 transgenic mice, and not in HPV negative, nontransgenic littermates. Therefore, treatment with vemurafenib alone, although sufficient to paradoxically activate the MAPK pathway in mouse dermis, and necessary for Ras wild-type tumorigenesis in K14-HPV16 mice, was not sufficient to initiate cSCC. Clearly loss of tumor suppressor
function is a minimum requirement for vemurafenib-induced cSCC.

HPV has been linked to cancer progression for decades due to the transformation potential of HPV oncogenes. HPV has been observed in both mucosal and cutaneous tumors, though the mechanism of oncogenesis likely differs. Cervical and head and neck cancers are clearly linked to \( \alpha \)-HPV genotypes, which exhibit mucosal tropism, whereas \( \beta \)-HPVs are more frequently associated with cutaneous disease. Although the mechanism of transformation for \( \beta \)-HPV is not fully understood, transgenic mouse studies demonstrate that keratinocyte-specific expression of either the HPV8 (\( \beta \)-HPV) or HPV16 (\( \alpha \)-HPV) genes can promote cSCC in the FVB/N background (14, 25). In addition, although expression of HPV transcripts can be seen in established cervical cancers, expression of HPV genes is often undetectable even in cSCC from HPV-positive patients (26),

![Image](image_url)

**Figure 4.** Cotreatment with PD0325901 prevents vemurafenib-induced cSCC. Mice were treated with 60 mg/kg vemurafenib daily, 3 mg/kg PD0325901 twice daily, 60 mg/kg vemurafenib daily + 3 mg/kg PD0325901 twice daily, or vehicle control. A, skin from mice was collected 10 hours after treatment and Dusp6 mRNA levels were measured by qPCR \( (*) P = 0.006; \ddagger\ddagger\ddagger P = 0.003; \ddagger\ddagger P = 0.002, n = 4, \) Student t test. B, frequency of histologic scoring \( n = 18, 23, 12, \) and 11 for vehicle, vemurafenib, PD0325901, and vemurafenib + PD0325901 cohorts, respectively; \( \ddagger P = 0.007; \ddagger\ddagger P = 0.006; \ddagger\ddagger\ddagger P = 0.0006, \chi^2 \) test.

![Image](image_url)

**Figure 5.** K14-HPV16 cSCCs are MEK dependent. Mice harboring spontaneous cSCC were treated with PD0325901 twice daily for 2 weeks. Tumor volume was measured at start and end of study. A, waterfall plot of tumor response to PD0325901, data expressed as% change of tumor volume after 2 weeks of treatment (horizontal line shows PR \( \geq 30%; \) CR = 100%). B, representative images of tumor responses before and after 2 weeks of treatment with 10 mg/kg PD0325901. C, representative images for pERK staining of cSCCs 5 hours after treatment with vehicle 3 mg/kg PD0325901 or 10 mg/kg PD0325901 \( n = 3 \) for each condition).
indicating that HPV does not play an active role in cSCC tumor maintenance. Data presented here demonstrate that a subsequent Ras mutation is also required for HPV16-driven tumorigenesis in the K14-HPV16 model (Fig. 3D). These data are consistent with the function of HPV16 E6 and E7 oncoproteins, which target P53 and pRB tumor suppressors to prevent the DNA damage response, and implicate HPV in cSCC initiation rather than tumor maintenance. Although it is unclear whether β-HPV initiate transformation through a similar mechanism, 27% (6/22) of cSCC from K14-HPV8 transgenic mice develop a spontaneous Hras mutation (27), compared with 11% (1/9) in the HPV16 model (Fig. 3D). Further investigation in β-HPV models may therefore reveal common mechanisms of oncogenesis.

These data therefore elucidate a synergy between premalignant, HPV-infected tissues, and paradoxical MAPK activation in patients treated with RAF inhibitors. HPV oncoproteins likely promote tumor initiation by increasing the frequency of premalignant mutations, whereas paradoxical activation of the MAPK pathway promotes both initiation and maintenance in patients treated with RAF inhibitors. Because most of the human population tests positive for cutaneous HPV (28), the majority of patients treated with RAF inhibitors are likely susceptible. Although only 18% to 30% of patients treated develop cSCC and/or keratoacanthoma (2, 3), these figures may underrepresent the frequency of RAF inhibitor-induced tumorigenesis. In a separate study of 42 patients, 100% presented with at least one adverse skin reaction, and the most common events were verrucous papillomas (79%; ref. 5). Patients who develop cutaneous tumors frequently acquire multiple primary lesions, which can affect entire limbs or chest (29, 30), a phenotype consistent with an infectious disease. Furthermore, the pathology of tumors is also not limited to cSCC and keratoacanthoma. Cases of pilaris-like eruptions, acantholytic dyskeratosis, verruous keratosis, cystic lesions, papules, verrucous papillomas, and basal cell carcinomas have been documented (29–33) but were not reported as adverse events in the clinical trials (2, 3). The diversity of cutaneous lesions observed in patients treated with RAF inhibitors may also reflect the presence of multiple oncoviruses, including Merkel cell polyomavirus (23), or multiple unrecognized HPV genotypes that each require MAPK activation for tumorigenesis. Disruption of ERK activation in these tissues by combining MEK and RAF inhibitors therefore seems to be an effective strategy to treat BRAFV600E-mutant melanomas, and to prevent spontaneous cutaneous tumors concurrently.

**Disclosure of Potential Conflicts of Interest**

M. Holderfield is a senior scientist at Frederick National Laboratory for Cancer Research, Leidos Biomedical Research, and a postdoctoral researcher at the Helen Diller Family Comprehensive Cancer Center. D. Stuart has ownership interests (including patents) in Novartis. C. Robert is a consultant/advisory board member for Roche, GSK, RMS, Merck, and Novartis. N. Pryer has ownership interest (including patents) in Novartis. F. McCormick is a coleader of the National RAS Project at Frederick National Laboratory. No potential conflicts of interest were disclosed by the other authors.

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