Contribution of Beta-HPV Infection and UV Damage to Rapid-Onset Cutaneous Squamous Cell Carcinoma during BRAF-Inhibition Therapy

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Abstract

Purpose: BRAF-inhibition (BRAFi) therapy for advanced melanoma carries a high rate of secondary cutaneous squamous cell carcinoma (cSCC) and risk of other cancers. UV radiation and α-genus human papillomavirus (HPV) are highly associated with SCC, but a novel role for β-genus HPV is suspected in BRAFi-cSCC. Cutaneous β-HPV may act in concert with host and environmental factors in BRAFi-cSCC.

Experimental Design: Primary BRAFi-cSCC tissue DNA isolated from patients receiving vemurafenib or dabrafenib from two cancer centers was analyzed for the presence of cutaneous oncogenic viruses and host genetic mutations. Diagnostic specimens underwent consensus dermatopathology review. Clinical parameters for UV exposure and disease course were statistically analyzed in conjunction with histopathology.

Results: Twenty-nine patients contributed 69 BRAFi-cSCC lesions. BRAFi-cSCC had wart-like features (BRAFi-cSCC-WF) in 22% of specimens. During vemurafenib therapy, BRAFi-cSCC-WF arose 11.6 weeks more rapidly than conventional cSCC when controlled for gender and UV exposure (P value < 0.03). Among all BRAFi-cSCC, ß-genus HPV-17, HPV-38, and HPV-111 were most frequently isolated, and novel β-HPV genotypes were discovered (CTR, CRT-11, CRT-22). Sequencing revealed 63% of evaluated BRAFi-cSCCs harbored RAS mutations with PIK3CA, CKIT, ALK, and EGFR mutations also detected.

Conclusions: We examined clinical, histopathologic, viral, and genetic parameters in BRAFi-cSCC demonstrating rapid onset; wart-like histomorphology; β-HPV-17, HPV-38, and HPV-111 infection; UV damage; and novel ALK and CKIT mutations. Discovered β-HPV genotypes expand the spectrum of tumor-associated viruses. These findings enhance our understanding of factors cooperating with BRAF inhibition that accelerate keratinocyte oncogenesis as well as broaden the knowledge base of multifactorial mediators of cancer in general. Clin Cancer Res; 1–11.

Introduction

Molecular inhibition of mutant BRAF protein in advanced melanoma with vemurafenib or dabrafenib (BRAFi) has improved patient survival but has also caused unanticipated adverse malignancies. BRAFi has been associated with cutaneous squamous cell carcinoma (cSCC; refs. 1–7) with unusually aggressive histopathologic potential (8), and even recurrence of KRAS-mutant colonic adenocarcinoma and emergence of undiagnosed chronic myelomonocytic leukemia (9, 10). The frequency of cSCC [including conventional cSCC (conv.-cSCC) and keratoacanthoma (KA)-type cSCC] during BRAFi is reported in 7.0% to 26.7% of patients (mean 9.7%; refs. 11, 13, 16, 17). In isolation, BRAFi-associated KA may complicate 5.8% to 14.3% of patients (mean 6.8%; refs. 11, 13, 14, 16, 17). With the addition of MEK inhibition, dabrafenib and trametinib (D+T) reduces the frequency of patients with cSCC/KA to 1.4% to 11.1% (D+T mean 2.2%; refs. 12, 19, 20) while combination of vemurafenib and MEK inhibitor cobimetinib reduces cSCC to 2.8% to 9.3% (mean 5.0%) and KA to 0.8% to 1.6% (mean 1.0%; refs. 17, 21).

The mechanism for cSCC development in otherwise healthy individuals is the result of UV radiation–induced transition mutations in host DNA (dipyrimidine or dipurine, e.g., A<->G or C<->T), which, if they occur in tumor suppressor genes, such as TP53, can cause protein inactivation, loss of cell cycle control, and cancer growth (22). UV radiation (particularly UVB) in conv.-cSCC induces mutations in HRAS (23). The limited studies on
BRAFi-induced cSCC (BRAFi-cSCC) mechanisms have employed focused “hot-spot” genetic analyses to begin to understand the multiplicity of variables that may contribute to adverse BRAFi-cSCC (13, 24–26). The data from these efforts demonstrate in part that upstream RAS mutations act in concert with paradoxical activation of MAPK signaling caused by BRAFi in 30% to 60% of lesions (13, 24–26). The remaining lesions appear to lack RAS mutation, and several studies (4–7, 27) have reported a morphologic pattern of wart-like features (WF) in BRAFi-cSCC, suggesting an additional possible contribution of human papillomaviruses (HPV) to this secondary disease.

SCC of the cervix is the prototypical virus-mediated epithelial cancer (28, 29). Infection by the α-genus of HPV (particularly genotypes HPV-16, -18, -31, -33) is most often associated with this disease, whereas condylomata of genital-mucosal sites are associated with the α-HPVs HPV-6 and HPV-11. More recently, infection by the α-genus HPVs has been implicated as a cause of a subset of head and neck SCC (28) and of both cutaneous warts or verrucae and squamous cell carcinoma of genitomucosal surfaces and cutaneous sites in immunocompromised patient populations (29).

Human papillomavirus 16 (HPV-16) oncoproteins E6 and E7 drive carcinogenesis in genital-mucosal sites. HPV-16 E6 and E7 oncoproteins inhibit cellular cell cycle–regulatory proteins TP53 and pRB, respectively, leading to abnormal cell proliferation, lack of normal differentiation, and prolonged survival. These features are all hallmarks of cancer.

Several studies attempting to link BRAFi-cSCC to α-HPV infection report negative results using immunohistochemical methods (5, 30, 31), based on detection of viral capsid protein L1 specific to α-HPVs (Dako K1H8 clone or ab2417; Abcam). A recent HPV DNA detection study of 7 patients contributing 9 biopsies confirmed the lack of infection with α-HPVs (32).

Immunocompromised patients suffer an increased burden of cSCC, suggesting a pathophysiology role for an infectious agent that may act with other host factors such as germline or somatic acquired genetic mutations. One promising candidate is β-HPVs, which contribute to cSCC in solid organ transplant patients, and several immunodeficiency states including epidermodysplasia verruciformis (EV), WHIM syndrome (Warts, Hypogammaglobulinemia, Infections, and Myelokathexis), and chronic lymphocytic leukemia/small lymphocytic lymphoma (see recent reviews in refs. 29, 33). The presence of wart-like histopathology in some BRAFi-cSCC, coupled with prior evidence for HPV-mediated carcinoma in healthy and immunosuppressed populations, suggests that β-HPVs contribute to the pathogenesis of BRAFi-cSCC.

Data support the linkage between BRAFi-cSCC and non-α-HPV infection. In genital-mucosal SCC, p16 is a tumor suppressor overexpressed during carcinogenic HPV infection. Strong p16 immunoreactivity is a surrogate for HPV infection in these sites. Two studies found strong p16 expression in the majority of SCC and KA lesions arising during BRAFi, arguing for a tumorigenic viral role (14, 27).

Recently, the β-genus HPV-17 and Merkel cell polyomavirus (MCPyV) were reported to coinfect one case of BRAFi-cSCC, again raising the possibility that β-HPV and other tumorigenic viruses participate in the development of BRAFi-cSCC (34). Further, in vitro evidence demonstrates cooperation between the paradoxical hyperactivation of the MAPK cascade with vemurafenib and β-HPV infection (35).

Finally, although RAS mutations have been reported frequently in BRAFi-cSCC, identical mutations have been detected in benign epithelial skin lesions (36), indicating that other factors in addition to RAS may be necessary in the oncogenicity of BRAFi-cSCC. The morphologic wart-like features we and others have observed in BRAFi-cSCC, combined with in vitro cooperation of vemurafenib MAPK signaling with HPV infection, overexpression of p16 in BRAFi-cSCC, and absence of α-HPV in BRAFi-cSCC, have served to establish the hypothesis that β-HPV infection may indeed be present in BRAFi-cSCC and serve a pathologic role. We sought to definitively examine and test for β-HPV and non-RAS genomic changes in primary clinical specimens from two independent cancer centers by employing central histomorphologic review, robust viral analysis, and next-generation sequencing to identify oncogenic changes in host DNA.

Materials and Methods

Case selection and patient protection

A retrospective analysis of previously procured diagnostic tissue samples was performed on patients treated with either study analogues of vemurafenib or dabrafenib with and without MEK inhibition by trametinib (D+T) in clinical trials. Patients were seen by either a medical oncologist (J.A. Joslin, D.B. Johnson, J.R. Infante) and/or dermatologist (B.R. Moody and W.A. Lumbang) in accordance with the clinical trial protocols (NCT00405587, NCT00880321, NCT01006980, NCT01107418, NCT01072175, NCT01271803, NCT01371271) for metastatic or unresectable melanoma. Clinical signs and symptoms prompted biopsy of skin lesions for histopathologic evaluation and diagnosis by a board-certified dermatopathologist (J.B. Robbins, J.P. Zwerner, A.S. Boyd). Twenty-nine patients had sufficient tissue from 69 biopsy specimens for morphologic examination and DNA extraction for viral genome analysis as described below. Biopsies were excluded if they had insufficient residual tissue for nucleic acid extraction and histologic confirmation of leisional tissue in the postsectioning H&E-stained slide, or if clinical parameters were incomplete. Re-excisions of lesions previously considered in the study were excluded to eliminate repetition bias. This study was approved by the Vanderbilt University Medical Center Institutional Review board for patient safety and research integrity (#111786, #130461).
Clinical parameters

The following patient-specific information was obtained for the study: gender, age, biopsy site, biopsy date, study medication, and date of initiation of therapy. UV radiation from sun exposure was estimated by the biopsy site. In men, the head and neck and upper extremities were considered “UV-exposed” (UV+). In women, in addition to the criteria for men, the upper chest, back, and lower legs were also considered “UV-exposed.” Biopsy sites that were not “UV-exposed” zones were considered “UV-protected” (UV−). Fitzpatrick skin-type was not utilized as a clinical criterion.

Pathology review

Histologic sections were prepared from formalin-fixed paraffin-embedded tissue and stained with hematoxylin and eosin under standard conditions. Lesional histomorphology was reviewed independently by four pathologists (D.N. Cohen, J.P. Zwerner, A.S. Boyd, J.B. Robbins), and descriptive and diagnostic consensus was reached. Histomorphology was determined to be conventional pattern of cSCC (conv.-cSCC), keratoacanthoma-type cSCC (cSCC-KA), or cSCC with wart-like features (cSCC-WF). Conv.-cSCC lesions are typified by keratinocyte proliferation in lobules with cytolagic and nuclear atypia and invasion into the dermis. Cutaneous SCC-KAs were composed of a crateriform eosinophilic keratinaceous core with a pushing border of atypical keratinocytes with papillary dermal invasion. When either cSCC-KA or conv.-cSCC revealed wart-like features that included overlying hyperkeratosis, hyperparakeratosis, papillomatosis, irregular nuclear contours, glassy nuclear inclusions, or koilocytic changes, the term cSCC-WF was applied.

DNA extraction

Nucleic acid was extracted from formalin-fixed paraffin-embedded tissue in the form of unstained slides or microtome-cut tissue curls and transferred to sterile microcentrifuge tubes. All sections were cut using a fresh knife-edge to prevent cross-contamination. Paraffin was removed via triplicate warmed xylene wash followed by centrifugation at 13,000 rpm for 5 minutes. Deparaffinized tissue was further washed 3 times with 100% ethanol followed by centrifugation at 13,000 rpm for 5 minutes. Tissues were dried at 37°C for 30 minutes, followed sequentially by proteinase digestion and DNA extraction using the QIAamp DNA Mini Kit per manufacturer’s instructions (Qiagen).

DNA quality determination and tumorigenic virus detection

The quality of the extracted DNA was assessed by β-globin reference gene PCR (37). HPV-DNA was amplified by PCR in the β-globin PCR-positive samples utilizing multiple nested primer systems designed for detection of broad ranges of HPV types (38–40). Isolation, cloning (TOPO TA cloning kit; Invitrogen Co.), and sequencing of putative HPV-PCR products were carried out as described (34). HPV sequence data were aligned and identified relative to known HPV DNA sequences available through GenBank (National Center for Biotechnology Information, National Institutes of Health, Bethesda, MD) using the BLAST program (41). A minimum of 5 clones was sequenced from the above TOPO-cloning procedure for HPV genotyping per specimen. Briefly, colony-purified E. coli isolates were grown to an optimal density, and plasmids containing viral sequences were purified for sequencing. PCR was employed to detect MCPyV in the β-globin PCR-positive specimens. MCPyV small T gene–based primers were used (34). PCR products were resolved in a 2.5% agarose gel and visualized with ethidium bromide for the expected 150 bp band. MCPyV-PCR products were extracted from the agarose, TOPO-cloned (as described above for HPV), and sequenced. Viral DNA sequences were subjected to BLAST alignment.

Host genetic analysis

Multiplex PCR was performed on previously extracted genomic nucleic acids, and next-generation sequencing was performed using a custom amplicon panel (Ion AmpliSeq Library 2.0, Ion OneTouch 200 Template v2, Ion PGM 200 Sequencing, Ion 318 Chip on Ion Torrent PGM using Torrent Suite 2.2 software). This assay interrogates regions of 35 known well-characterized cancer-related genes (Supplementary Table S3). DNA quality was assured by qPCR for β-globin before library preparation. Sequence reads were analyzed in a Clinical Laboratory Improvement Amendments (CLIA)-approved laboratory environment by determining target coverage, strand bias, read length, quality score, and other parameters. Suitable reads were aligned to the reference human genome GRCh37 using CLCbio Genomics Workbench and CLCbio Genomics Server. Data were annotated using COSMIC and dbSNP databases. Allele frequency cutoff of ≥10% and/or ≥100× coverage with approximately equal strand bias were predefined inclusion criteria. Sanger sequencing was performed using standard methods with primers directed at corresponding oncoprotein mutations in PCR amplicons (Supplementary Table S3; Integrated DNA Technologies).

Statistical analysis

A P value less than or equal to 0.05 was defined as statistically significant before all analyses. The time from initiation of study medication (vemurafenib, dabrafenib, D+T) to biopsy of cSCC, termed hereafter as "latency," was calculated by subtracting the initiation date from the biopsy date. To compare the latency of lesion onset between groups, we employed the Wilcoxon rank sum or Kruskal–Wallis nonparametric tests using R (v.3.0.3 & 3.1.0, The R Foundation for Statistical Computing). We studied the relationship between latency and morphology of vemurafenib-treated lesions while adjusting for UV exposure status and gender using a linear mixed-effects model with random intercept. The latencies of multiple lesions per patient were not considered as independent. Patient 6 on dabrafenib therapy was unusual with 7 lesions arising quickly. These displayed unique spindle-cell squamous carcinoma histomorphology and have been reported elsewhere (8). Another patient (#19) treated with D+T also had lesions arising quickly (Supplementary Table S1). These 2 patients were omitted from multivariate model analyses as the number of lesions and rapidity of onset indicated these cases to be outliers.

Results

Demographics of the study

Histomorphologic and viral analyses were performed on 69 cSCCs obtained during diagnostic procedures for 29 patients with BRAF V600-mutant melanoma enrolled in clinical trial studies of vemurafenib (N = 22 patients, n = 53 lesions), dabrafenib (N = 4 patients, n = 10 lesions), or combination of dabrafenib with trametinib (MEK inhibitor; D+T, N = 3 patients, n = 6 lesions; Table 1). Twenty-one men and 8 women provided 52
Table 1. Patient and specimen parameters and tumorigenic virus detection

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**Table 1.** Patient and specimen parameters and tumorigenic virus detection (Cont’d)

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<td>V</td>
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<td>69</td>
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<td>Y</td>
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<td>Clavicle</td>
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<td>69</td>
<td>M</td>
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<td>N</td>
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<td>V</td>
<td>12</td>
<td>KA</td>
<td>11, 124, FA14, SIBX18</td>
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**Figure 1.**
Sun-exposed sites most frequently harbor BRAFi-cSCC. Distribution and histopathology of all adverse cSCC lesions from male (blue, left) and female (rose, right) patients with advanced melanoma treated with BRAFi. Conventional-cSCC (circles), well-differentiated keratoacanthomatous-type SCC (squares), and conv.-cSCC or cSCC-KA with wart-like features (cSCC-WF, stars).

**Primary melanoma genetics and therapy parameters**

The majority of patients had metastatic or unresectable melanoma with mutations in the BRAF gene that resulted in valine (V) to glutamate (E) amino acid changes at position 600 (V600E, N = 26 patients). Three total patients (one with each mutation) had mutations that caused V600K (valine to lysine), V600D (valine to aspartate), and V600R (valine to arginine) contributing 2, 2, and 3 lesions, respectively (Table 1).

**Histomorphology of BRAFi-cSCC**

Features contributing to histomorphologic classification are reviewed in the Materials and Methods section. Thirty-six percent (n = 25) of lesions had conventional cSCC morphology (BRAFi-conv.-cSCC [Fig. 2B and E]), 42% (n = 29) were BRAFi-cSCC with keratoacanthomatous features (BRAFi-cSCC-KA [Fig. 2F]), and 22% (n = 15) were BRAFi-cSCC with wart-like features (BRAFi-cSCC-WF [Fig. 2A, C and D]). The morphologic distribution was similar to previously reported studies (1, 2, 37, 38, 1, 2, 11, 13, 25, 26, 42).

**Latency to development of lesions**

For all patients, the median latency was 13 weeks with a maximum of 77 weeks and minimum of 1 week (Table 1). The latency also depended on therapy administered. There were 53 lesions from patients on vemurafenib, with 10 lesions from dabrafenib-treated patients and 6 lesions from patients on D+T. The mean latency regardless of histopathology while on vemurafenib (22 weeks) or Dab (10.9 weeks) was significantly shorter than D+T (32.1 weeks, P = 0.02) including patients #6 and #19.

The median latency for patients on vemurafenib therapy (N = 22 patients, n = 53 lesions) was shorter in UV-exposed sites (median, 13 weeks, n = 33) compared with UV-protected regions (median, 25.5 weeks, n = 20, P = 0.010), supporting the hypothesis that UV DNA damage may accelerate BRAFi-cSCC. Further, the BRAFi-conv.-cSCC morphotype showed significantly more rapid onset from UV-exposed sites (Fig. 3; median, 13 weeks, n = 12) compared with UV-protected (median, 27 weeks, n = 7, P = 0.016). Cutaneous BRAFi-SCC-KA did not have statistically significant UV-exposure latency differences: 21.5 versus 12.5 weeks UV-protected versus UV-exposed (Fig. 3; n = 10 vs. n = 12, respectively).

**NOTE:** Patient (P) identification sample number, male (M) or female (F) gender; left (L) or right (R) laterality; UV radiation-exposed skin (Y); UV radiation-protected skin (N).

*bPrimary melanoma tumor mutation: BRAF valine at amino acid 600 (V600) mutated to glutamate (E), aspartate (D), lysine (K), or arginine (R).
*bTherapy (Tx): V = vemurafenib, D = dabrafenib, D+T = dabrafenib with trametinib.
*bHistomorphology of conventional (C) cSCC with KA and invasive spindle-SCC (KA*; ref. 8) or with wart-like features (Fig. 2B, C, D, E, F).
*bHPV genus identified or not detected (ND).
*bMCPyV identified: yes (Y), no (N) or not performed (NP).
Latency to development of lesions by morphologic type

The morphologic subtype (BRAFi-conv.-cSCC, BRAFi-cSCC-KA, or BRAFi-cSCC-WF) correlated with latency. Using a multivariate model, we compared the mean latency difference between lesions with different morphology adjusted for UV exposure and gender. The latency in BRAFi-cSCC-WF decreased significantly by 11.6 weeks (Fig. 3, P = 0.03) compared with BRAFi-conv.-cSCC for vemurafenib-treated patients’ lesions with the same UV exposure and gender. Taken together, in the context of morphology regardless of UV exposure, vemurafenib-treated patients tended to show longer median latency with cSCC-KA (13.0 weeks) and conv.-cSCC (18.5 weeks) compared with cSCC-WF (11 weeks); however, this difference did not meet statistical significance (P = 0.14). The D+T group showed no cSCC-WF and a majority of conv.-cSCC. The median latency for the D+T group was 52 weeks (n = 4, after excluding patient #19).

Spectrum of HPV infection during BRAFi

Cutaneous β-genus HPVs were identified in the BRAFi-cSCC of all morphologic types, with the predominant subtypes being HPV-38 (n = 28), HPV-17 (n = 21), and HPV-111 (n = 11; Table 1; Supplementary Fig. S1; Supplementary Table S1). Thirty-nine BRAFi-cSCC lesions were infected by more than one HPV genotype. β-HPVs previously associated with cSCC, HPV-5 (n = 0) and HPV-8 (n = 1), were infrequently found, and none of the subtypes associated with condylomata or SCC in genital-mucosal surfaces were identified (HPV-6, -11, -16, -18, etc.; Table 1).

Seventeen samples were negative for virus detection in our study (Table 1) and were processed on the same day using the same method as samples that were HPV or MCPyV positive. This reduces the likelihood of knife-blade contamination (43), supporting the reliability of the method to reduce false-positive testing.

Putative novel HPV genotypes identified

Three viral nucleic acid sequences from patients P005, P022, and P028 were unique, each with less than 90% homology to
previously reported HPV genotypes in GenBank (NCBI; Supplementary Fig. S1 and Supplementary Table S1). These were designated as putative novel β-genus HPVs CTR, CRT-22, and CRT-11. Multiple alignment techniques demonstrated partial similarity of the novel HPV-CTR with previously described HPV-118, implying genus- or subgroup-level relatedness, whereas novel HPVs CTR-11 and CRT-22 demonstrate more distant relatedness to their closest known relatives, implying that they may represent novel HPV genotypes (Supplementary Fig. S2 and Supplementary Table S2; refs. 40, 44). The phylogenetic relatedness of HPV isolates in this study is compared in Supplementary Fig. S1 with closest known relatives, implying that they may represent novel HPV-CTR with previously described HPV-118, implying genus- or subgroup-level relatedness, whereas novel HPVs

### Table 2. Host genetic mutations and polymorphisms in known cancer-causing genes in BRAFi-cSCC.

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Tx</th>
<th>Type</th>
<th>UV</th>
<th>HRAS</th>
<th>KRAS</th>
<th>PIK3CA</th>
<th>ALK</th>
<th>EGFR</th>
<th>CKIT</th>
<th>PDGFRA</th>
<th>Total</th>
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<tr>
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<td>Dab</td>
<td>WF</td>
<td>Y</td>
<td>Q61L</td>
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<td></td>
<td>M541L</td>
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<td>P006</td>
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NOTE: Oncogenic amino acid changes and SNPs in oncogenes detected in BRAF inhibition-associated cSCCs during therapy for advanced melanoma. RAS mutations predominate with additional PIK3CA, ALK, and EGFR mutations detected using Ampliseq (IonTorrent) with Sanger sequencing (italic) methods. Horizontal lines delineate samples from individual patients.

### Discussion

BRAFi therapy for melanoma improves patient survival, but is complicated by secondary cSCC (BRAFi-cSCC) of unclear etiology and unknown biologic potential (8). These BRAFi-cSCCs are clinically relevant, as they can limit duration of therapy for some patients. BRAFi acts in concert with other factors to increase the frequency of cutaneous, hematologic, and visceral cancer by paradoxical hyperactivation of the MAP kinase cascade. Although BRAFi-cSCC is a recognized complication of BRAFi, few clinical trials have employed central dermatopathology review of biopsy material. In this work, we present the largest clinical and molecular study published to date of skin cancers arising related to BRAF inhibition for melanoma. We have performed a detailed study of the secondary SCC latency during BRAFi, including 29 patients and 69 biopsy tissue samples from two cancer centers on one of three current drug regimens (vemurafenib, dabrafenib, or D-T) for metastatic or unresectable advanced melanoma.

The histomorphologic subtypes of BRAFi-cSCC include conventional, BRAFi-cSCC-KA, and BRAFi-cSCC-WF. We identified approximately 20% of BRAFi-cSCC lesions as BRAFi-cSCC-WF similar to previous reports (5–7). The time from initiating therapy to diagnosis of cSCC (latency) was significantly associated with histopathology of BRAFi-cSCC. The latency of BRAFi-cSCC-WF was 11.6 weeks shorter that BRAFi-conv.-cSCC (P = 0.03) when the two groups were compared while controlling for gender and UV radiation exposure using a multivariate model (Fig. 3). A
that UV exposure did not significantly influence BRAFi-cSCC-KA or -WF may suggest that other factors play a more dominant role in these lesions compared with the UV radiation pathogenesis via TP53 inactivation in conventional-type cSCC (22).

The latency to develop SCC was also influenced by therapy. The differential latency to initial cSCC onset by therapy type (vermurafenib < dabrafenib < D+T) and morphology (conv. > KA > WF) may have pathophysiologic consequences. We observed that lesions with wart-like features frequently have earlier onset than KA or conventional-type cSCC, extending previous work where the comparison did not reach statistical significance (14). The combination of D+T results in decreased incidence of cSCC (19); our study shows a trend toward prolongation of latency (52 weeks vs. 16.3 weeks for vermurafenib), which correlates with the effect previously proposed (19). The relatively small sample size of D+T and dabrafenib-only groups in this study precludes definitive demonstration of significance. Recent studies indicate the BRAFi can inhibit apoptosis through target INK pathway effects that may explain the incomplete elimination of adverse cSCC during D+T (46).

Because so many (>20%) of BRAFi-cSCCs have wart-like features reminiscent of viral-driven processes, and because previous studies had failed to isolate α-HPVs from these samples, we took a broadly sensitive approach to identify other, more novel tumorigenic viruses, including non–α-HPV and MCPyV.

The HPVs have close homology within the L1 capsid gene that can be amplified by multiple primer sets: PGMY, GP5+/GP6+, FAP, EV-HPV (38–40, 47). In our study, several lesions with wart-like features were negative with one primer set and positive with another primer set, demonstrating the need for a redundant PCR approach not reported in prior studies. We found no oncogenic α-HPV genotypes, in agreement with a recent report (32), and the only α-HPV found was a non-oncogenic genotype (HPV-7) coinfected with two β-HPVs (HPV-38 and HPV-17).

Under normal conditions, the β-HPV genotypes commensally reside in the human skin, and associate with cSCC in immunocompromised patients (28). β-HPV-5, HPV-8, and other β-HPVs are also found in cSCC from immunocompromised renal transplant and EV patients (28). Although reports of HPV in cSCC from otherwise healthy patients also reveal γ-HPV genotypes (48), few patient lesions in our study demonstrated γ-HPV infection: patients #1 and #28 each had one BRAFi-cSCC infected by HPV-65 and FA15, respectively, and each BRAFi-cSCC with a γ-HPV infection was also β-HPV coinfected (Table 1).

The most frequently detected HPV genotypes in the present study (HPV-38, HPV-17, and HPV-111) are very closely related and as such subclassified together as β2 species that are phylogenetically distant from the α-genus HPV that infect genital/mucosal surfaces and cause cervical SCC (Supplementary Fig. S1). This may suggest that the cutaneous tropism and oncogenic activity of E6 and E7 under BRAFi paradoxical MAPK cascade activation may be restricted to a particular biologic subset of β-HPV types related to and including HPV-38, HPV-17, and HPV-111.

β-HPV has been associated with cSCC from healthy patients, with frequent serologic evidence and intralapial HPV nucleic acid detected; however, the frequency of detection with different methods is highly variable, thus making study comparison difficult (49, 50). Of interest, β2-HPVs have previously been associated with cSCC from extensively sun-exposed sites (51). Examination of cSCC from otherwise healthy individuals identified HPV-38 and HPV-17 in 13.5% (12 of 89) of infected cSCCs using similar methods to our study (52). In our study, HPV-38 and HPV-17 comprised a significantly higher rate of total infections: 40% (49 of 123, P ≤ 0.0001 (2.7e–05), Fisher exact test (two-sided); Table 1). Further, studies have demonstrated transforming properties of in vitro cell culture and in vivo mouse models of HPV-38 E6 and E7 putative oncoproteins (53, 54) that have enhanced in vitro transforming activity in the presence of BRAFi (35).

MCPyV is a significant causative factor in the cutaneous neuroendocrine carcinoma known as Merkel cell carcinoma. In this study, MCPyV was found in 13 BRAFi-cSCC lesions, often with coinfection of β-HPV (9 of 13 lesions). MCPyV infection and expression of the large T antigen have been found to interrupt the cell cycle–regulatory protein pRB (55). Coincidentally, the HPV E7 protein also inhibits pRB (28). The detection of MCPyV in these lesions, concurrent with HPV and in isolation, reinforces a prior observation of HPV-17 and MCPyV coinfection (34). MCPyV has also been detected in cSCC from immunocompetent patients and healthy individuals; however, the causative or commensal nature of MCPyV in this setting is unclear (56). Because of the frequent coinfection of MCPyV and HPV in this study, it is difficult to make a definitive statement regarding the causal nature of MCPyV infection in BRAFi-cSCC.

Of the lesional biopsies that were available for next-generation sequencing and had detectable mutations, all but 4 were infected by β-HPV. Canonical cancer-associated HRAS (Q61, G12) and KRAS (G12) amino acid mutations were confirmed in 5 of 8 lesions (62.5%, Table 2), similar to the frequency reported in other studies (13, 24–26). The present study adds two examples of HRAS mutation coincident with HPV infection (HRAS G12T with β-HPV36b—P006; KRAS G12D β-HPV 17 and β-HPV 151—P144). To our knowledge, this has only been observed once before (57).

We also demonstrate the presence of relevant mutations in other cancer-associated proteins not previously reported in BRAFi-cSCC. EGFR, ALK, CKIT, and PIK3CA mutations are most commonly associated with lung, breast, endometrial, and colorectal carcinomas, gastrointestinal stromal tumors, and lymphomas. Mutations in these oncoproteins are not frequently detected in cSCC. PIK3CA mutations have been detected in two spontaneous cSCCs and one KA lesion (24) and in two BRAFi-cSCCs (26). One EGFR R108K mutation has been reported in a single BRAFi-cSCC (26). We report for the first time to our knowledge an ALK F1174L mutation, two tumors with CKIT M541L mutations, and an EGFR V769M mutation in BRAFi-cSCCs.

Prior mechanistic studies of conv.-cSCCs have demonstrated a significant role for UV-B via increased rate of mutation in tumor suppressor TP53 (23). We also found in BRAFi-conv.-cSCC that UV exposure significantly increased the rate of onset (decreased latency). By contrast, BRAFi-cSCC-WF does not show a significant effect of UV radiation on latency in the present study, indicating a possible independence from UV induction in this subtype of BRAFi-cSCC. Although the number of MCPyV-infected samples in this study was too low to draw specific conclusions, UV-B has been associated with induction of MCPyV small T antigen expression (58). A larger sample size may reveal an interplay of UV radiation, β-HPV, and MCPyV coinfection during BRAFi-cSCC from primary patient cancer tissues.
An alternate hypothesis, which is intriguing but not possible to evaluate rigorously with our study, states that BRAFi-cSCC is the same lesion that has been sampled at different time points in a unique tumor progression, beginning with wart-like features and culminating with keratoacanthomatus or conventional cSCC morphology. Lesions biopsied early in this life history would morphologically have wart-like features and lesions biopsied later in development would have fewer wart-like features. Further study into the life history of BRAF inhibition–induced carcinogenesis using the cSCC as a model system may prove or disprove this hypothesis.

Although we included 69 biopsies from 29 patients, the study size was limiting for the non-vemurafenib groups to preclude extensive analysis of dabrafenib with and without the MEK inhibitor trametinib (D+T). Additional biopsies were not identified for patients on the vemurafenib and cobimetinib MEK inhibition combination therapy. Further, the limitation of sample size prevents demonstration of statistical significance for several parameters suggestive but not definitive of relevance to BRAFi-cSCC carcinogenesis in our study, including coinfection of β-HPV and MCPyV with and without UV exposure and RAS gene mutation. The primary measure in the study of time from therapy initiation to lesion is limited by patient presentation. This bias would overestimate the true time to onset as the initiating genetic events leading to cancer precede epidermal overgrowth and thus precede discomfort, bleeding, or other causes that then draw patients’ attention and therefore provider intervention. Ampliseq by Ion Torrent is a versatile platform for focused amplicon-based next-generation sequencing. However, the pipeline has occasional failure. NGS and analysis in our experiments did report HRAS Q61L for P040 that on Sanger was confirmed as Q61R. In addition, the annotation algorithm misidentified a G12 change in sample P006 that was confirmed G12T by Sanger. This correction was required because the CLCbio database contains duplicate entries, both single and double base mutations, for this codon that was misidentified in the algorithm. A possible double PIK3CA mutation resulting in A1046T (P070) was suggested by the NGS that was not confirmed by Sanger (Table 2). Also, CKIT, EGFR, and ALK mutations were within Ampliseq study parameters; however, there was insufficient biopsy DNA for further Sanger confirmation.

In conclusion, β-HPV infection is frequent in BRAFi-cSCC, and BRAFi-cSCC-WF is statistically associated with rapid-onset lesions. The large number of novel HPV genotypes identified in these lesions needs more complete genetic study to begin to understand putative E6/E7 oncoproteins in nonmucosal HPV infection, but indicates the possibility of subgroup-specific biologic factors in BRAFi oncogenesis. Further in-depth study into the host genetic patterns of BRAFi-cSCC-WF and UV-damage host genetic models in conjunction with HPV and MCPyV large and small T antigens may provide sufficient power for analysis. These novel studies will likely shed new insight into the pathogenesis of SCC as well as multifactorial models for cancer in general.

Disclosure of Potential Conflicts of Interest

P.K. Chandra reports receiving speakers bureau/honoraria from Pfizer. Y. Shyr is a consultant/advisory board member for Aduro Biotech, Inc. and GlaxoSmithKline. J. Infante is a consultant/advisory board member for Genentech and GlaxoSmithKline. No potential conflicts of interest were disclosed by the other authors.

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Administrative, technical, or material support (i.e., reporting and organizing data, constructing databases): S.K. Lawson, H.P. Nguyen, J.A. Sosman

Study supervision: S.K. Tyring, J.R. Infante

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Clinical Cancer Research

Contribution of Beta-HPV Infection and UV Damage to Rapid-Onset Cutaneous Squamous Cell Carcinoma during BRAF-Inhibition Therapy


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