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Presence of Human Polyomavirus 6 in Mutation-Specific BRAF Inhibitor–Induced Epithelial Proliferations

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Abstract

IMPORTANCE—A frequent adverse effect of mutation-specific BRAF inhibitor therapy is the induction of epithelial proliferations including cutaneous squamous cell carcinomas. To date, the only factor identified contributing to their development is the activation of the mitogen-activated signal transduction cascade by mutations in the *RAS* genes. However, these mutations explain only 60% of the tumors; hence, it is important to identify what is causing the remaining tumors.

OBJECTIVE—To test for the presence of human papillomaviruses (HPVs) and the recently identified human polyomaviruses (HPyVs), Merkel cell polyomavirus (MCPyV), and trichodysplasia spinulosa–associated polyomavirus (TSPyV), as well as HPyV-6, HPyV-7, HPyV-9, and HPyV-10, in epithelial proliferations occurring after BRAF inhibitor therapy to determine whether these oncogenic viruses may contribute to BRAF inhibitor–induced skin tumors.

DESIGN, SETTING, AND PARTICIPANTS—Retrospective study at a university hospital in Austria of epithelial proliferations that developed in patients with melanoma after initiation of treatment with the BRAF inhibitor vemurafenib. Samples were analyzed for (1) presence of the most frequently observed *RAS* mutations by SNaPshot technology, (2) detection of the viruses by

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Study concept and design: Schrama, Pastrana, Becker.

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real-time polymerase chain reaction, and (3) presence of capsid proteins of the most abundantly detected virus by immunohistochemical analysis.

MAIN OUTCOMES AND MEASURES—*RAS* mutational status, as well as HPV and HPyV presence, in BRAF inhibitor–induced epithelial proliferations.

RESULTS—Eighteen biopsy samples from 6 patients were retrieved from our hospital's archive. We identified *RAS* mutations in 10 (62%) of the 16 samples with clear results. DNA of HPyV-9, HPyV-10, and TSPyV were virtually absent in the samples. MCPyV DNA was present in 13 of 18 samples, and HPV, HPyV-6, and HPyV-7 DNA were present in all samples. In general, the amount of DNA encoding the latter viruses was rather low, with the exception of HPyV-6 in several samples of 1 individual patient. Notably, the relevance of the presence of HPyV-6 in the epithelial proliferation was underlined by immunohistochemical detection of the core protein VP1 of HPyV-6.

CONCLUSIONS AND RELEVANCE—The presence of both high HPyV-6 DNA load and VP1 protein suggests that polyomaviruses may contribute to the epithelial proliferations observed in patients receiving BRAF inhibitor therapy, albeit the relative impact as compared with that of *RAS* mutations appears circumstantial.

Melanoma, originating by transformation of melanocytes, is one of the most aggressive skin cancers. Indeed, treatment of stage IV melanoma was frustrating for a long time.[1] Recently, however, several effective therapies including immune checkpoint–modulating antibodies or targeted signal transduction inhibitors have been developed. In this regard, the small-molecule kinase inhibitor vemurafenib was approved by the US Food and Drug Administration and the European Medical Agency for treatment of metastatic melanoma in 2011, and several other drugs followed or are currently being evaluated for approval.[2] Vemurafenib, as well as the also approved dabrafenib, specifically inhibits valine 600 (V600)-mutated BRAF[3,4] encoded by the proto-oncogene *BRAF*. Mutations in this gene are present in the tumors of approximately 50% of patients with melanoma,[5] and treatment of susceptible tumors results in response rates of up to 70%. Unfortunately, responses to V600 mutation–specific BRAF inhibitors are generally temporary, with a median time to relapse of approximately 6 months.[6,7]

Pathological skin proliferations such as keratoacanthomas, cutaneous squamous cell carcinomas (SCCs), or even new primary melanomas have been reported as adverse effects of BRAF inhibitor therapy. For example, in a phase 3 study of vemurafenib, 26% of treated patients developed at least 1 keratoacanthoma or SCC.[6] *RAS* mutations are assumed to be the major factor contributing to the pathogenesis of BRAF inhibitor–induced epithelial proliferations; indeed, *RAS* mutations are present in up to 60% of these proliferations.[8,9] BRAF inhibitors can stimulate the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) pathway in *RAS* mutated and/or *BRAF* wild-type cells, a notion also known as paradoxical activation of the MAPK/ERK pathway.[10,11] Consistent with this mechanism, BRAF inhibitor treatment accelerated tumor development in a murine tumor model based on a *RAS* mutation.[9] To date, apart from *RAS* mutations, not many predisposing factors for BRAF inhibitor–induced epithelial proliferations have been reported. For the multikinase inhibitor sorafenib, *TP53* and *TGFRB1* mutations have been

reported, and for vemurafenib *TP53* mutations were identified in a few cases.[9,12] Thus, the mutational spectrum observed in BRAF inhibitor–induced epithelial proliferations is smaller than in spontaneous cutaneous SCC.[8] However, because in approximately 40% of BRAF inhibitor–induced epithelial proliferations *RAS* is not mutated, other factors are likely to contribute to their pathogenesis. In this regard, human papillomaviruses (HPVs) have been suspected to be involved in the development of cutaneous SCC.[13] Hence, infectious agents such as oncogenic viruses may also be involved in BRAF inhibitor–induced epithelial proliferations. Notably, a recent case report identified HPV and Merkel cell polyomavirus (MCPyV) DNA in a cutaneous SCC that developed after BRAF inhibitor therapy.[14]
In the present study, we tested for the presence of HPV and human polyomaviruses (HPyVs), ie, MCPyV and trichodysplasia spinulosa–associated polyomavirus (TSPyV), as well as HPyV-6, HPyV-7, HPyV-9, and HPyV-10, in a series of BRAF inhibitor–associated epithelial proliferations.

Methods

Patients

The study was approved by the institutional review board of the Medical University of Graz. Because of the retrospective nature of the study, the institutional review board did not require patient consent for the molecular workup of samples that had already been obtained from patients for therapeutic purposes. A total of 18 archived paraffin-embedded tumor samples from 6 patients with stage IV melanoma were collected from the Department of Dermatology. All tumors had been excised for therapeutic reasons. The presence of transformed keratinocytes in the selected samples was confirmed by a dermatopathologist. All tumors had developed after the patients received vemurafenib treatment for advanced melanoma.

DNA Isolation and Virus Detection

Genomic DNA was isolated from serial sections using the peqGOLD tissue DNA mini kit (PEQLAB). Real-time polymerase chain reaction (PCR) was performed for the detection of HPV, MCPyV, and TSPyV, as well as HPyV-6, HPyV-7, HPyV-9, and HPyV-10. Primer and PCR conditions for HPV, MCPyV (LT3), and long interspersed nuclear elements (LINEs) have been described previously.[15,16] For the other viruses, new primers located in the small T antigen-coding regions were designed with Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/): HPyV-6_st_Fw TAG CAC TTG TAG CAC CAG, HPyV-6_st_Rv ATG CCT TCA TTG CCT TCT, HPyV-7_st_Fw TCT AAC CTT ATG CTG TAT G, HPyV-7_st_Rv GGT AGA GAT GAA GTC AAG, TsPyV_sT_Fw CCT TGA TTA ACT CTC AGA TA, TsPyV_sT_Rv GAC TTA TAT TGC TTG GAT TC, HPyV-9_st_Fw GAA ATC CAA ACC AAA GTA AGT A, HPyV-9_st_Rv CCT ATA ATA AGC CCA GTT GT, HPyV-10_st_Fw ACT TCT CCT TCT CTT CTA AGC, HPyV-10_st_Rv GCA ATA TAA ATA CAG GCA GAC TT. Quantitative PCR was performed with LuminoCt mastermix (Sigma Aldrich) in 20-μL volume reactions with 1 μL DNA according to the manufacturer's instructions on a StepOnePlus real-time PCR system (Life Technologies). The relative presence of virus genome in the samples was determined by the ΔΔCt method. A highly repetitive DNA element, LINE1, served as endogenous control. The values for

MCPyV detection in a Merkel cell carcinoma cell line (MKL-2 with mean [SD], 1.7 [0.5] copies/cell[17]) served as common calibrator, allowing comparison of the relative presences of all other viruses.

RAS Mutation Analysis

For the detection of hot-spot mutations of *HRAS* (OMIM 190020), *KRAS*, and *NRAS* the highly sensitive *RAS* SNaPshot multiplex assay was applied as described.[18,19] In brief, after the multiplex amplification of exons 1 and 2 of *HRAS*, *KRAS*, and *NRAS*, a SNaPshot multiplex set with primers designed to anneal directly adjacent to the potential mutation site was performed. These primers contain poly(dT) tails of different length, allowing the probes to be distinguished by size. The set covers bases 34, 35, 37, 181, and 182 of *HRAS*, 34, 35, and 181 of *KRAS*, and 34 and 182 of *NRAS*. Products of PCR were subsequently separated by capillary electrophoresis on an automatic ABI sequencer (Life Technologies).

Immunohistochemical Analysis

For visualization of the VP1 major capsid protein of HPyV-6 in formalin-fixed paraffin-embedded tissue samples, slides were deparaffinized and rehydrated. After antigen retrieval with citrate buffer, pH 6.0 (Dako), and a wash with phosphate-buffered saline (PBS), peroxidase-blocking solution (Dako S2023) was applied for 10 minutes at room temperature. After 2 washing steps with PBS, the slides were incubated with mouse monoclonal antibodies 6V12 or 6V32, which were elicited against HPyV-6 virus-like particles using previously reported methods.[20] 6V12 (isotype IgG3)is specific for HPyV-6VP1, whereas 6V32 (isotype IgG1) cross-reacts with HPyV-7 VP1. Neither monoclonal antibody is reactive with the VP1 protein of MCPyV. After primary antibody binding, the slides were washed twice again with PBS and then incubated with biotinylated secondary antibody (K5003A, Dako) for 30 minutes at room temperature; after 2 more washing steps with PBS, streptavidin horseradish peroxidase (K5003B, Dako) was applied for 20 minutes at room temperature. Slides were washed twice in PBS and stained with ImmPACT NovaRED (Vector Laboratories) for 8 minutes at room temperature. After another wash in PBS, slides were counterstained with hematoxylin (Dako), rinsed in water, dehydrated, and mounted in Tissue-Tek Glas mounting medium (Sakura Finetek).

Results

Sample Overview

For the present study, we selected 18 BRAF inhibitor–induced epithelial proliferations, ie, 1 acanthoma, 3 keratoacanthomas, and 14 cutaneous SCCs, from 6 different patients with stage IV melanoma. Because of their size, 4 of these samples were divided into 2 blocks and were each analyzed individually. The presence of individual atypical keratinocytes (the 1 acanthoma) or apparently cancerous cells (all others) in the samples used for molecular workup was confirmed by a pathologist (L.C.). Excision of the skin lesions occurred between 28 and 146 days after the start of BRAF inhibitor therapy. Details on patients and samples are given in the Table.

RAS Mutation Analysis

Because BRAF inhibitor–induced keratoacanthomas and cutaneous SCC frequently harbor *RAS* mutations, we first analyzed the presence of such mutations. To this end, we performed multiplex SNaPshot testing on DNA. This panel covers the majority of *RAS* mutations (21 of 24) previously described in BRAF inhibitor–induced epithelial proliferations.[8,9] For 16 of 18 samples, we obtained a clear result (Figure 1). HRAS Q61L was the most frequent mutation, present in 6 samples. In 1 sample we detected a heterogeneous mutation pattern with HRAS and KRAS G12D (sample4). Each of the respective mutations was found in an additional sample. KRAS G12C was detected in 1 sample. In total, 10 of our 16 samples (62%) harbored a *RAS* mutation. In 6 of the samples, we did not find any of the analyzed *RAS* mutations.

Presence of Viral DNA

Recently, a case report demonstrated the presence of HPV17 and MCPyV in a BRAF inhibitor–induced SCC.[14] To extend this observation, we performed real-time PCR to determine the presence of HPV, MCPyV, and TSPyV, as well as HPyV-6, HPyV-7, HPyV-9, and HPyV-10, in the BRAF inhibitor–induced epithelial proliferations. The assay for HPV is based on the use of degenerate primers targeting the highly conserved HPV E1 region, theoretically detecting almost any HPV type.[15] The primers for the HPyVs were located in the T antigen region coding of each virus. Thus, even when the virus is integrated, this genomic region is unlikely to be lost by integration. To allow a comparison among all different viruses, the relative presence was calculated in comparison with the values obtained for MCPyV for MKL-2, a Merkel cell carcinoma cell line containing approximately 1.7 MCPyV copies/cell.[17] Human papillomavirus was detected in all samples, ranging from 0.002 to 0.046 relative presence; 14 samples had values larger than 0.005 (Figure 2A).The presence of HPyV-9 was detected only in sample 14, with a relative presence of 0.001, whereas TSPyV and HPyV-10 were detectable in a few samples with low relative presence values (<0.005) (Figure 2E and 2F). In contrast, MCPyV and HPyV-7 were present in the majority of samples, although the relative presence value for MCPyV was rather low, ranging from 0.0007 to 0.007 (Figure 2B). For HPyV-7, most positive samples also had low values (13 samples <0.005), but in 3 samples the values were greater than 0.01 and in 1, greater than 0.1(Figure 2D).The greatest values of relative presence, however, were observed for HPyV-6. Indeed, the majority of samples had values greater than 0.005. Moreover, in 3 samples of patient 1 we determined values greater than 0.1 and in an additional sample even a value of 1.28 relative presence (Figure 2C).

Presence of HPyV-6 VP1 Protein

Because, on a DNA level, HPyV-6 was the most abundant virus in the analyzed BRAF inhibitor–induced epithelial proliferations, we performed immunohistochemical analysis to visualize the HPyV-6 VP1 protein. To this end, we used 2 different anti-VP1HPyV-6 monoclonal antibodies (6V32 and 6V12), which gave similar results although 6V32 generated more background staining. Individual VP1-positive cells were identified. Indeed, in sample 2 for which we had determined the highest relative presence of HPyV-6, the tumor

harbored many VP1-expressing cells (Figure 3A–C), whereas in samples from other patients HPyV-6 VP1 expression was not detected at all (Figure 3D–F).

Discussion

Mutation-specific BRAF inhibitor therapy alone, or in combination with other small-molecule kinase inhibitors, is an effective therapy for patients with stage IV BRAF-mutated melanoma, even for those with high tumor burden.[21] Therapy with BRAF inhibitors, however, is associated with the induction of new skin lesions ranging from benign seborrheic keratosis or well-differentiated SCC to even melanoma.[22,23] The underlying mechanism seems to be a process known as paradoxical MAPK/ERK pathway activation: binding of BRAF inhibitors to wild-type BRAF leads to dimer formation, which subsequently can be activated by RAS, thus triggering the MAPK/ERK pathway.[10,24] Paradoxical MAPK/ERK pathway activation has been observed in vitro, leading to increased proliferation of wild-type BRAF keratinocytes, and in vivo; eg, after BRAF inhibitor treatment the frequency of phosphorylated ERK–positive keratinocytes in human and murine skin increases.[12,25] Notably, however, in mouse dermis BRAF inhibitor treatment alone seemed not to be able to initiate cutaneous SCC.[9,25] This observation further sustains the hypothesis that RAS activation is a prerequisite for BRAF inhibitor–induced epithelial proliferation. Indeed, in therapy-associated cutaneous lesions of patients receiving BRAF inhibitor therapy, a frequency of *RAS* mutations ranging from 16% to 60% has been reported.[8,9,25] In our sample cohort, we detected *RAS* mutations in 10 of 16 samples (62%), with 1 sample harboring 2 different *RAS* mutations.

Thus, the majority of the BRAF inhibitor–induced epithelial proliferation in our study can be explained by *RAS* mutations. However, in approximately 40% of lesions, ie, in accordance with the frequency reported in the literature, *RAS* mutations did not explain the epithelial proliferations. Given the verruca-like appearance of many of these lesions, as well as the association of HPV seropositivity with cutaneous SCC,[26] transforming viral proteins may be an alternative co-factor for BRAF inhibitor–induced epithelial proliferation. Consequently, we tested for the presence of HPV and several polyomaviruses. Of the recently newly described polyomaviruses,[27–32] only MCPyV and TSPyV have been associated with a disease.[27,33–35] Nevertheless, seropositivity of these cutaneous polyomaviruses is high, ranging from 66% to 81% in adults, demonstrating their infectious potential.[36]

Among the analyzed viruses, HPyV-9, HPyV-10, and TSPyV were virtually absent in all lesions. This is in accordance with the fact that these viruses seem only rarely to reside in the skin.[37] In contrast, those viruses regularly present in normal skin, ie, HPV, MCPyV, HPyV-6, and HPyV-7,[28,37–39] were present in most (MCPyV) or all samples (HPV, HPyV-6, HPyV-7), although the amount of viral DNA in each case was generally low. Notably, however, for HPyV-6 we determined a pronounced viral load in several samples from the same patient. The detection of viral DNA of viruses regularly present in the skin is not a sufficient argument for a possible role of that virus in the pathogenesis of the skin lesions that it was detected in. However, the protein expression of HPyV-6 VP1 in the BRAF inhibitor–induced epithelial proliferation with the highest prevalence suggests active viral

replication. Because this study was based on tissue specimens removed for diagnostic and/or therapeutic purposes, we did not have normal skin samples from each patient to establish and compare the presence of HPyV-6 with that in normal tissue in the same patient. Notably, studies trying to determine possible associations of viral infection with carcinogenesis are always hampered by the fact that viral activity might be necessary only at tumor initiation and not for maintenance of the tumor, the latter being typically the period when samples are obtained.[40] For example, despite the epidemiologic evidence for an association of HPV and cutaneous SCC, expression of HPV genes is only rarely detectable in cutaneous SCC.[41]

For BRAF inhibitor–induced epithelial proliferations, the presence of viral DNA of HPV, and in 1 case report, of MCPyV, has been reported.[14,25] In the present study, we were also able to detect viral DNA for HPV (without verifying the types) and MCPyV, and additionally HPyV-6 and HPyV-7; however, only for HPyV-6 did they occur in an amount suggesting a possible function. Detection of viral gene products in BRAF inhibitor–induced epithelial proliferations, however, is an extremely rare event; in 1 study of 6 SCCs and 10 verruca-like acanthomas, HPV VP1 expression was detected only in 1 verruca-like acanthoma.[22] Similarly, Ganzenmueller and colleagues[42] were not able to detect viral transcripts in any lesion from 5 different patients by means of next-generation sequencing. However, the functional relevance of transforming virally encoded proteins for BRAF inhibitor–induced skin carcinogenesis has been demonstrated in a murine model based on expression of the early coding sequences from HPV-16 in keratinocytes in which vemurafenib treatment increased SCC incidence from 22% to 70%.[25] Interestingly, all the SCCs developing in the control group harbored *RAS* mutations; in contrast, in the vemurafenib-treated mice only 45% of the SCCs harbored a *RAS* mutation.

Conclusions

Currently, the role of cutaneous viruses as co-factor for BRAF inhibitor–induced epithelial proliferation is not clear. On the one side are reports demonstrating the presence of viral DNA in tissue samples; conversely, on the other side detection of viral gene transcription is largely absent. Here we identified not only several samples from 1 individual patient containing high amounts of HPyV-6 DNA but also samples with HPyV-6 VP1 protein expression, suggesting a contribution of HPyV-6 to the pathogenesis of that BRAF inhibitor–induced epithelial proliferation. However, these tumors also harbored *RAS* mutations, making it impossible to judge the scale of the impact of HPyV-6 encoded proteins on BRAF inhibitor–induced epithelial proliferation.

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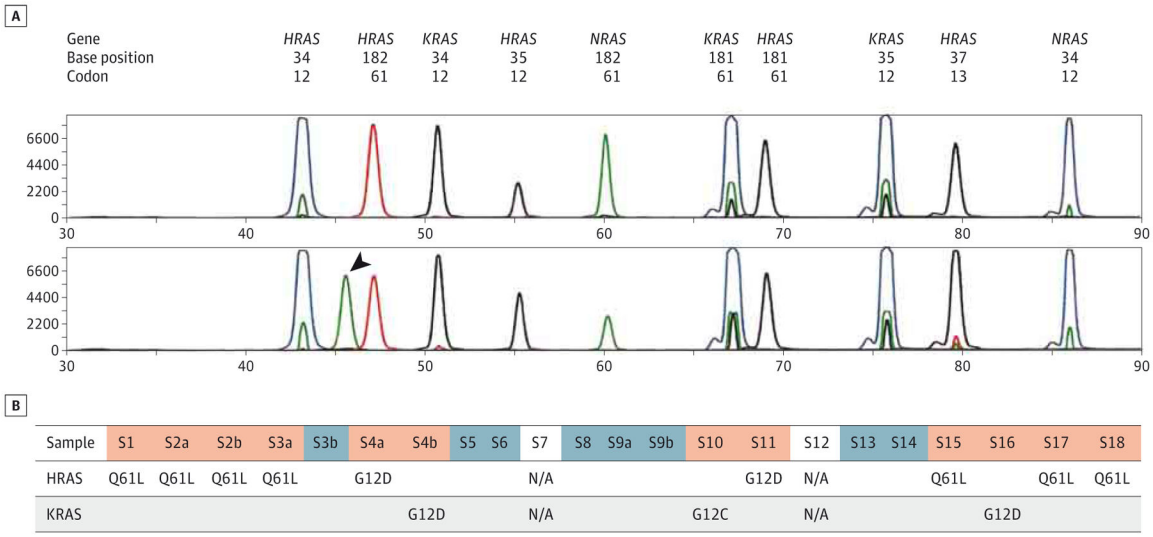

Figure 1. HRAS Q61L *RAS* Mutation

DNA from epithelial proliferations occurring in patients receiving BRAF inhibitor therapy was analyzed for the presence of *RAS* mutations by means of SNaPshot technology. A, Each peak in the chromatogram represents a hot-spot mutation site. For each peak, the respective gene, base position, and the encoded codon are shown. The upper chromatogram is an example of a sample (sample 8) in which all analyzed bases are wild type. In the lower chromatogram the A to T mutation at base position 182 of the *HRAS* gene is present (arrowhead), leading to the HRAS Q61L mutation. B, Table of the mutations present in the analyzed samples. Sample numbers with mutations are on a red background; those without, blue. N/A indicates the 2 samples that did not yield a clear result. *NRAS* mutations were not detected in any sample.

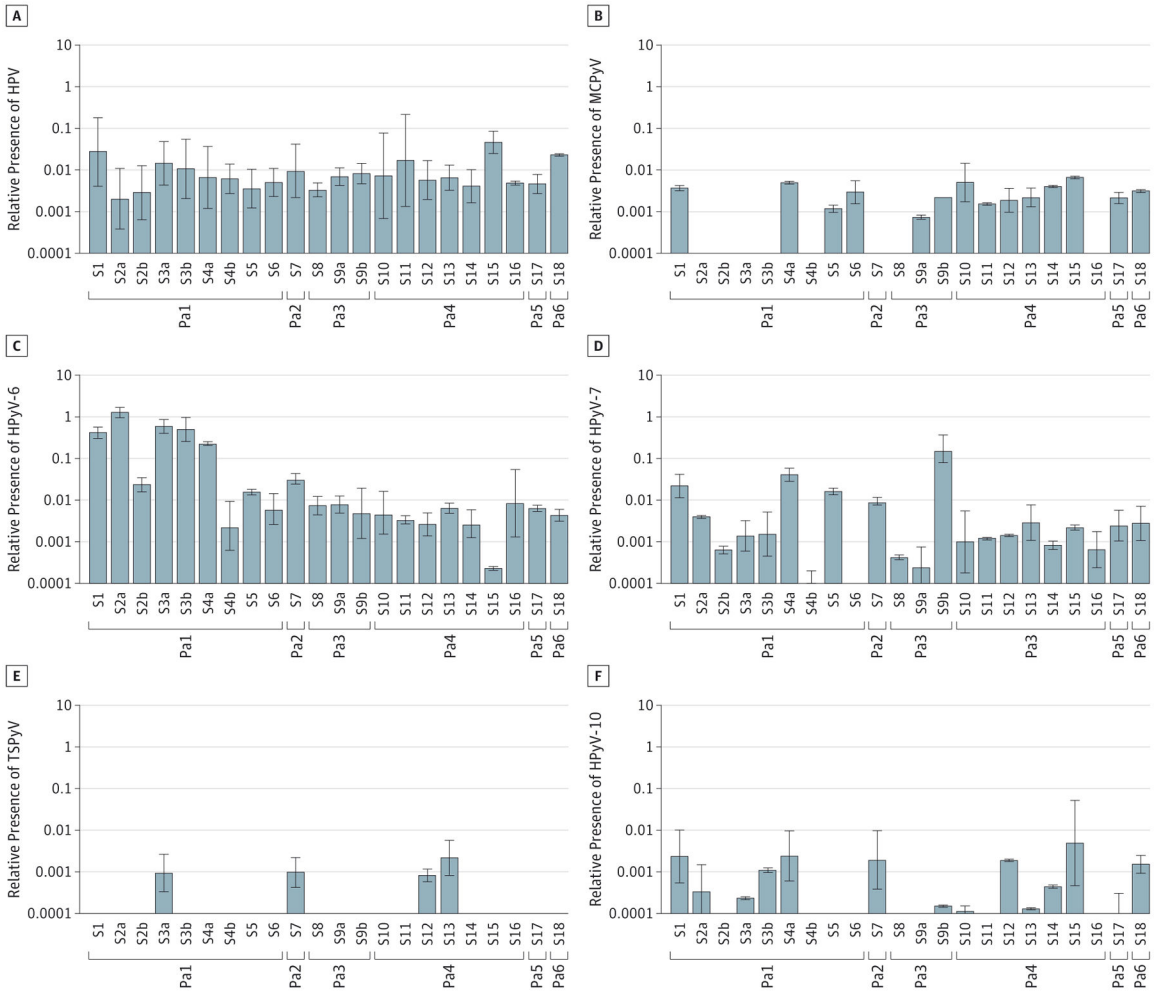

Figure 2. Relative Presences of Human Papillomavirus (HPV), Merkel Cell Polyomavirus (MCPyV), Human Polyomavirus Type 6 (HPyV-6), HPyV-7, Trichodysplasia Spinulosa-Associated Polyomavirus (TSPyV), and HPyV-10

Real-time polymerase chain reaction was performed for the detection of HPV (A), MCPyV (B), HPyV-6 (C), HPyV-7 (D), TSPyV (E), and HPyV-10 (F). The MCPyV values obtained for the Merkel cell carcinoma cell line MKL-2 served as calibrator for all calculations. The bars indicate mean relative presences, and the error bars, the minimum and maximum values. Pa indicates patient, and S, sample.

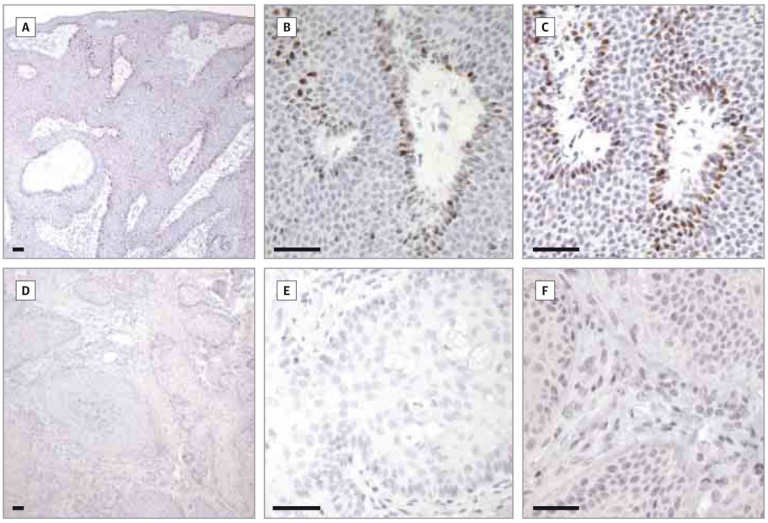

Figure 3. Expression of Human Polyomavirus Type 6 (HPyV-6) VP1 Protein

Formalin-fixed paraffin-embedded sections were stained with NovaRED and counterstained with hematoxylin for HPyV-6 VP1 protein (original magnification ×10 [A and D]; original magnification ×40 [B, C, E, and F]). Two different antibody sera (6V12 [A, B, D, and E] and 6V32 [C and F]) obtained from immunized mice were used. Depicted are the results for sample 2 (A–C) and sample 15 (D–F). The scale bar indicates 50 μm.

Table.

Patient and Sample Characteristics

Patient No./Sex/Age, y[a]	Sample No.	Excision, Days[b]	Localization	Diagnosis
1/F/70s	1	28	Neck	Well-differentiated SCC
	2a, 2b	28	Thoracic	Well-differentiated SCC
	3a, 3b	57	Pretibial	SCC
	4a, 4b	57	Presternal	Well-differentiated SCC, keratoacanthoma type
	5	57	Eyebrow	Well-differentiated SCC
	6	57	Knee	Well-differentiated SCC, keratoacanthoma type
2/F/50s	7	109	Shoulder	Well-differentiated SCC
3/F/40s	8	83	Neck	Well-differentiated SCC
	9a, 9b	111	Neck	Well-differentiated SCC, keratoacanthoma type
4/F/50s	10	35	Preauricular	Well-differentiated SCC
	11	35	Nasolabial	Uncommon virus acanthoma with atypical keratinocytes
	12	35	Nose	Well-differentiated SCC
	13	49	Lower jaw	Well-differentiated SCC
	14	49	Forehead	Well-differentiated SCC
	15	49	Forehead	Well-differentiated SCC
	16	49	Forehead	Well-differentiated SCC
5/F/50s	17	56	Upper eyelid	Hyperplastic actinic keratosis with transformation into SCC
6/F/50s	18	146	Capillitium	Well-differentiated SCC

Abbreviation: SCC, squamous cell carcinoma.

[a]Age when patient started BRAF inhibitor therapy.

[b]Days between start of BRAF inhibitor therapy and excision of skin lesion.