Human Cancer Biology

Activation of PI3K Signaling in Merkel Cell Carcinoma

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Abstract

Purpose: Merkel cell carcinoma (MCC) is an aggressive cutaneous neuroendocrine tumor, often metastatic at presentation, for which current chemotherapeutic regimens are largely ineffective. As its pathogenesis is still unknown, we hypothesized that deregulation of signaling pathways commonly activated in cancer may contribute to MCC tumorigenesis and may provide insights into targeted therapy approaches for this malignancy.

Experimental Design: We retrospectively profiled 60 primary MCC samples using a SNaPshot-based tumor genotyping assay to screen for common mutations in 13 cancer genes.

Results: We identified mutations in 9 (15%) MCC primary tumors, including mutations in TP53 (3 of 60) and activating mutations in the PIK3CA gene (6 of 60). Sanger sequencing of the primary MCC tumors detected one additional PIK3CA mutation (R19K) that had not been previously described in cancer. Merkel cell polyoma virus (MCPyV) was detected in 38 (66%) MCC cases and patients with MCPyV-positive cancers showed a trend toward better survival. With one exception, the presence of MCPyV and activating mutations in PIK3CA appeared mutually exclusive. We observed that signaling through the PI3K/pAKT pathway was active in one MCPyV-positive and in all MCPyV-negative MCC cell lines, as evidenced by AKT phosphorylation. Importantly, the presence of a PIK3CA-activating mutation was associated with sensitivity to treatment with ZST474, a specific phosphoinositide 3-kinase (PI3K) inhibitor, and to NVP-BEZ235, a dual PI3K/mTOR inhibitor, targeted agents under active clinical development.

Conclusions: PI3K pathway activation may drive tumorigenesis in a subset of MCC and screening these tumors for PIK3CA mutations could help identify patients who may respond to treatment with PI3K pathway inhibitors.

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Introduction

Merkel cell carcinoma (MCC), originally defined as a “trabecular carcinoma of the skin,” is an aggressive neuroendocrine skin tumor first described almost 40 years ago (1). Although initially believed to derive from Merkel cells, the slowly adapting mechanoreceptors located in the basal cell layers of the epidermis and the only cutaneous cells containing electron-dense neurosecretory granules (2), the histogenesis of MCC is still disputed with some evidence pointing to a pluripotent stem cell origin (3, 4).

MCC is quite rare, with an estimated incidence of 3 new cases per million people per year in the United States (5). The incidence of MCC has been on the rise (6), and increases with age, exposure to ultraviolet radiation, and with immunosuppression, being up to 15 times higher in patients with HIV, organ transplants, and hematologic malignancies such as multiple myeloma, non–Hodgkin lymphomas, and chronic lymphocytic leukemias (7, 8). The most common primary site of presentation is the skin of the head and neck, followed by the skin of the upper extremities, lower extremities, and trunk. Interestingly, albeit to a much lower extent (<1%), MCC may also arise in extracutaneous sites including the parotid gland, submandibular glands, the nasal cavity, and the lymph nodes (9).

MCC tends to present as a firm, flesh-colored, red- or blue-livid painless nodule, which is fast growing and can rapidly spread to regional lymph nodes and to distant sites (10, 11). The extent of disease at presentation is a strong predictor of survival, with a 10-year survival rate of 70% for patients with localized disease, which sharply drops to 20% if the patients present with distant metastases (9).
MCCs metastasize and at that stage treatment options are infected MCC (20, 21).

of MCPyV-positive MCC cell lines, suggesting that T antigen getting the viral T antigens result in cell-cycle arrest and death MCC (18, 19). Furthermore, knockdown experiments targeting LT and small T antigen (ST) viral sequences are significantly expressed in the binding motif of LT is spared and both LT and small T of the presence of prematurely truncating mutations in the (14). Although integrated MCPyV cannot replicate, because viral infection precedes clonal expansion of the cancer cells humans (16, 17), and evidence of a possible mechanistic role for MCPyV in MCC tumorigenesis is starting to emerge. First, MCPyV DNA was shown to integrate into the genome of MCC tumors in a clonal pattern, strongly suggesting that viral infection precedes clonal expansion of the cancer cells (14). Although integrated MCPyV cannot replicate, because of the presence of prematurely truncating mutations in the viral large T antigen (LT) DNA, the retinoblastoma (RB)-binding motif of LT is spared and both LT and small T antigen (ST) viral sequences are significantly expressed in MCC (18, 19). Furthermore, knockdown experiments targeting the viral T antigens result in cell-cycle arrest and death of MCPyV-positive MCC cell lines, suggesting that T antigen expression may play a role in the oncogenesis of MCPyV-infected MCC (20, 21).

Unfortunately, despite optimal early management, many MCCs metastasize and at that stage treatment options are very limited and typically fail. Thus, new mechanistic and therapeutic insights would be very valuable. In an attempt to identify novel oncogenic pathways involved in MCC tumorigenesis and with the hope of uncovering therapeutically relevant mutations that could help improve management of patients with MCCs, we retrospectively profiled 60 primary tumors using the previously described SNaPshot tumor genotyping assay. This test screens for hotspot mutations across 13 cancer genes and has been used at our institution for more than 2 years to guide therapy for patients with solid tumors (22).

Material and Methods

Patient specimens

This study was conducted according to the principles expressed in the Declaration of Helsinki. Work on human tissue was conducted on excess archival human material after obtaining approval from the Massachusetts General Hospital (MGH; Boston, MA) Institutional Review Board (protocol reference: 2008-P-002165; MGH). We collected a retrospective cohort of patients with a diagnosis of MCCs who had undergone surgery at MGH between January 1995 and September 2010. Sixty patients were identified, for whom formalin-fixed, paraffin-embedded (FFPE) tumor specimens suitable for molecular analysis could be obtained. The patients’ medical records were reviewed to collect demographic data and relevant information about clinical history, presentation, management, and outcome. Stage at diagnosis was determined according to the American Joint Committee on Cancer (AJCC) guidelines (23).

Cell lines

We established a permanent cell line, MGH-mcc1, derived from one of the primary tumors in our cohort (MCC-12). Briefly, fresh tumor (isolated within 1 hour after surgery) was isolated from a left axillary dissection and washed with 50 mL of wash buffer (Dulbecco’s Modified Eagle’s Media + 2% FBS + gentamicin and fungizone). Tissues were minced using sterile crossed blades to produce small (<1 x 1 mm³) pieces under sterile conditions. Tumor pieces were left to grow in 6-well plates with 17% FBS + RPMI + 1-glutamine+ antibiotic/antimycotic. After approximately 4 to 6 weeks, single colonies were subcloned and pooled to generate the MGH-mcc1 cell line. Human MCC cell lines MCC13, MCC26, MKL-1, MKL-2, and UISO-mcc1 had been previously described (18, 24–27). All MCC cell lines were maintained in RPMI-1640 medium supplemented with 10% FBS.

MCPyV detection by PCR

Testing for the presence of MCPyV was carried out using 2 previously described sets of primers MCPYV1 (28) and MCPYV2 (29), both of which yield short PCR products (109 and 195 bp, respectively) being well suited for testing poor quality DNA derived from FFPE tissue. In short, 20 ng of tumor DNA was subjected to PCR amplification using the
primers described below and the Platinum Taq Kit (Invitrogen) for 40 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 68°C for 1 minute. To ensure that a negative signal was not an artifact related to DNA integrity, in addition to the MCVPS1 (forward, 5'-TCAGCGTCCCAGGCTTCAGA-3'; reverse, 5'-TGGTCTCCTCCTGTACTG-3') and MCPyV (forward, 5'-ACTTGGGAAAGTTTTGACTGGTGGCAA-3'; reverse, 5'-GGGCCTCGTCAACCTAGATGGGAAAG-3'), primer sets, which detect viral-specific sequences in MCPyV-positive tumors (28, 29), we set up a parallel amplification reaction to detect KRAS exon 2 (forward, 5'-tcattatttttatattagGCCTGCTG-3'; reverse, 5'-agaatggtcctgcaccagtaa-3'), which yielded a 185-bp fragment for all tested samples. Because of the lack of sufficient tumor material for 2 cases, MCPyV analysis was conducted on 58 of the 60 MCC specimens.

### Tumor mutational analysis

Histologic examination of hematoxylin and eosin-stained slides derived from FFPE was conducted by a pathologist and assessed for the presence of tumor. Available tumor tissue was manually microdissected or cored from the paraffin block using a 1.5-mm dermal punch. Total nucleic acid was extracted from FFPE material using a modified FormaPure System (Agencourt Bioscience Corporation). Mutational analysis for a panel of 13 cancer genes, which included BRAF, EGFR, KRAS, NRAS, PIK3CA, and TP53 (Supplementary Table S1), was conducted as previously described (22). This analysis used a recently developed tumor genotyping protocol based on the SNaPshot multiplex platform (Applied Biosystems) that efficiently detects multiple mutations in tumor DNA extracted from FFPE tissue (22).

### Table 1. Baseline characteristics of patients with MCC

<table>
<thead>
<tr>
<th></th>
<th>All patients, N = 60</th>
<th>PIK3CA mutational status</th>
<th>MCPyV status*</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Positive, n = 7</td>
<td>Negative, n = 53</td>
<td>Positive, n = 38</td>
</tr>
<tr>
<td>Age (y)</td>
<td></td>
<td></td>
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<tr>
<td>Median</td>
<td>73</td>
<td>68</td>
<td>73.5</td>
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<tr>
<td>Range</td>
<td>44–94</td>
<td>44–94</td>
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<td>Sex</td>
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<td>38 (63)</td>
<td>7 (100)</td>
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<td>Female</td>
<td>22 (37)</td>
<td>0 (0)</td>
<td>20 (53)</td>
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<tr>
<td>Immunosuppression</td>
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<td></td>
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<td>1 (14)</td>
<td>5 (13)</td>
</tr>
<tr>
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<td>52 (87)</td>
<td>6 (86)</td>
<td>33 (87)</td>
</tr>
<tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>15 (25)</td>
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<td>12 (32)</td>
</tr>
<tr>
<td>II</td>
<td>13 (22)</td>
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<td>9 (24)</td>
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<td>III</td>
<td>23 (38)</td>
<td>3 (43)</td>
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<td>IV</td>
<td>9 (15)</td>
<td>2 (29)</td>
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</tr>
<tr>
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<td>Head and neck</td>
<td>27 (45)</td>
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<td>3 (8)</td>
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<tr>
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<td>3 (43)</td>
<td>16 (42)</td>
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<td>2 (29)</td>
<td>8 (21)</td>
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<tr>
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<td>11 (18)</td>
<td>1 (14)</td>
<td>6 (16)</td>
</tr>
<tr>
<td>Unknown</td>
<td>15 (25)</td>
<td>1 (14)</td>
<td>8 (21)</td>
</tr>
</tbody>
</table>

NOTE: All values are expressed as n (%).
*Because of limited amount of tumor, we were unable to determine MCPyV status for 2 cases.
*Stage I vs. II–IV.
*Stage I–III vs. IV.
Sequence analysis of mutational hotspots mapping within the PIK3CA adapter–binding, helical and kinase domains was conducted by direct sequencing of M13-tagged PCR products generated using the following primer pairs: PIK3CA exon 1, 5'-agtgaacagcgcagtgctgcctgcaatcagccaaat-3' (forward) and 5'-caggaaacagctatgaccagctgccagagatcagccaaat-3' (reverse); exon 9, 5'-TGTAAAGCAGCGCCAGTttggaacgagggaaa-3' (forward) and 5'-CAGGCCAGTctgtgaatccagagggaaa-3' (reverse); and 5'-caggaaacagctatgaccagctgccagagatcagccaaat-3' (reverse); exon 20, 5'-agtgaacagcgcagtgctgcctgcaatcagccaaat-3' (forward) and 5'-caggaaacagctatgaccagctgccagagatcagccaaat-3' (reverse). Thermocycling was conducted at 95°C for 8 minutes, followed by 45 cycles of 95°C for 20 seconds, annealing at 58°C (exon 1 and 20) or at 68°C (exon 9) for 30 seconds and 72°C for 1 minute, and one last cycle of 72°C for 3 minutes. Sequencing was conducted using the incorporated M13 primer tag, as previously described (22).

The epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor 2 (HER2) gene copy number were assessed by FISH as previously reported (30).

Immunoblotting
Cells were prepared and lysed as previously described (31). Proteins were resolved using the NuPAGE Novex Midi Gel system on 4%–12% Bis-Tris Gels (Invitrogen) and transferred to nitrocellulose membranes. Immunoblotting was conducted using the following antibodies according to the manufacturers’ recommendations: total Akt (Santa Cruz); phospho-AKT (308) and phospho-AKT (473; both from Cell Signaling); and actin (Sigma-Aldrich).

Flow cytometry
Fluorescence-activated cell-sorting analysis was conducted on a BD LSR III (Becton Dickenson). For apoptotic measurements, experiments were carried out as previously described (31). The annexin Cy5 was from BioSource International. Experiments were carried out in triplicate and error bars represent SDs.

Statistical analysis
Fisher exact test and Wilcoxon rank-sum test were used to assess the association of MCPyV or PIK3CA genotype status with baseline patient characteristics. Overall survival was calculated from the date of initial diagnosis of MCC to the date of death and was censored at the date of last follow-up for patients who were alive. Overall survival was estimated by the Kaplan–Meier method, and the difference between MCPyV groups was assessed using proportional hazards regression to adjust for the independent effects of stage and nodal status. The data analysis was computed by SAS 9.2 (SAS Institute), and all P values were based on a 2-sided hypothesis.

Results
MCC cohort characteristics
To study the genetic etiology of MCCs, we identified surgical pathology specimens from patients who underwent resection of their tumors at the MGH from 1995 to 2010. We identified 60 cases of MCCs with sufficient tissue for molecular analysis.

In our cohort, the median age at disease presentation was 73 years (range, 44–94 years), 38 (63%) patients were male, and 8 (13%) were immunocompromised because of HIV infection, solid organ transplant, or leukemia (chronic lymphocytic leukemia or chronic myelogenous leukemia; Table 1). The most common site of presentation was the head and neck (45%) followed by the extremities (35%), with only 5 (8%) tumors presenting in the trunk. In 7 (12%) cases, the primary tumor site was unknown, as these patients first presented with nodules in the lymph nodes or with disseminated metastatic disease. Approximately, half (n = 28) of the patients presented with either stage I (25%) or stage II (22%) disease, 23 (38%) patients presented with stage III, and a minority (15%) had stage IV disease. Disease staging was determined according to the AJCC guidelines (23).

Of the 60 MCC specimens available for molecular analysis, 40 (67%) were primary tumors and 20 (33%) were recurrences. Median tumor size was 2.2 cm (range, 0.5–12.5 cm). Representative images of the MCC cases are illustrated in Fig. 1 and include the classical neuroendocrine morphology (Fig. 1A) and the typical, although nonspecific, immunohistochemistry with cytoplasmic positivity for synaptophysin (Fig. 1B) and perinuclear dot-like positivity for CK20 (Fig. 1C). Additional histopathologic features are outlined in Table 2 and included an unusual spindle shape morphology observed in only a subset of cases (5 of 60, approximately half) of the patients presented with either stage I (25%) or stage II (22%) disease, 23 (38%) patients presented with stage III, and a minority (15%) had stage IV disease. Disease staging was determined according to the AJCC guidelines (23).

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Figure 1. Histology and immunohistochemistry of MCCs. A, hematoxylin and eosin staining illustrating a population of monomorphic, noncohesive round cells with a high nuclear to cytoplasmic ratio and finely granular “salt and pepper” chromatin. B, positive staining for the neuroendocrine marker synaptophysin. C, CK-20 dot-like perinuclear staining. Additional features observed in some tumors: (D) spindle morphology (arrow), (E) large zone of necrosis, and (F) prominent nucleoli (arrow). Scale bar, 50 μm.
The presence of MCPyV correlated with better survival

To determine the prevalence of MCPyV in our cohort, we used PCR-based protocols and 2 sets of primers that had been previously optimized to detect viral load in nucleic acid extracted from archival material (28, 29). We detected MCPyV in 38 (66%) of 58 tumors (Supplementary Table S2). Patients with MCPyV-positive MCCs were significantly more likely than MCPyV-negative patients to be female (53% vs. 5%, \( P < 0.001 \)) and to have a lower stage disease at presentation (I–III vs. IV; 95% vs. 70%, \( P = 0.016 \); Table 1).

MCPyV-positive tumors were significantly less likely than MCPyV-negative MCCs to show evidence of pleiomorphic spindle cell morphology (3% vs. 20%, \( P = 0.044 \)) or to be associated with necrosis (24% vs. 65%, \( P = 0.004 \); Table 2).

Stratification of the overall patient population according to viral status revealed a statistically significant improvement in the overall survival (\( P = 0.023 \)) and recurrence-free survival (\( P = 0.027 \)) of MCPyV-positive patients. After adjusting for the independent effects of stage and nodal status, patients with MCPyV-positive tumors showed a trend toward longer overall survival (\( P = 0.065 \)), when compared with patients with no detectable virus (Fig. 2; Supplementary Table S3).

SNPshot genotyping detected PIK3CA-activating mutations in MCC

SNPshot mutational profiling of the 60 specimens identified point mutations in 9 (15%) cases (Supplementary Table S4). Three (5%) tumors harbored mutations in the TP53 tumor suppressor gene (R248P, R248W, and R273H). Interestingly, 6 (10%) MCCs had activating mutations in the p110\( \alpha \) subunit of the PIK3CA gene (Fig. 3; Supplementary Table S4). Five tumors harbored mutations located within the helical domain of PIK3CA, including 3 cases with the E542K substitution, and one case each with the E545K- and the Q546K-activating mutations. The sixth case carried the H1047L somatic mutation located within the kinase domain of PIK3CA.

All of the variants detected by SNPshot were confirmed by conventional Sanger sequencing, which was also used to screen MCCs for the presence of additional mutations within the PIK3CA gene. This effort identified one extra variant located within the adaptor-binding domain of...

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**Table 2. Pathology of MCC cases**

<table>
<thead>
<tr>
<th>Specimen size (cm)</th>
<th>All patients, ( N = 60 )</th>
<th>PIK3CA mutational status</th>
<th>MCPyV status(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive, ( n = 7 )</td>
<td>Negative, ( n = 53 )</td>
<td>( P )</td>
</tr>
<tr>
<td>Specimen size, cm</td>
<td>Median 2.2</td>
<td>8.0</td>
<td>2.0</td>
</tr>
<tr>
<td>% Necrosis</td>
<td>0.5–12.5</td>
<td>3.5–12.5</td>
<td>0.5–8.0</td>
</tr>
<tr>
<td>Specimen size, cm</td>
<td>&lt;2</td>
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<td>0 (0)</td>
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<tr>
<td>2–12.5</td>
<td>31 (52)</td>
<td>6 (86)</td>
<td>25 (47)</td>
</tr>
<tr>
<td>Unknown</td>
<td>8 (13)</td>
<td>1 (14)</td>
<td>7 (13)</td>
</tr>
<tr>
<td>% Necrosis</td>
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<td>0</td>
</tr>
<tr>
<td>% Necrosis</td>
<td>0–95</td>
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<tr>
<td>Nucleoli visible</td>
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<td>37 (62)</td>
<td>2 (29)</td>
</tr>
<tr>
<td>5%–95%</td>
<td>23 (38)</td>
<td>5 (71)</td>
<td>18 (34)</td>
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<td>6 (86)</td>
</tr>
<tr>
<td>Positive</td>
<td>18 (30)</td>
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<td>17 (32)</td>
</tr>
<tr>
<td>Positive/pleiomorphic</td>
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<td>5 (71)</td>
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<tr>
<td>Vascular invasion</td>
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<td>2 (29)</td>
<td>3 (6)</td>
</tr>
<tr>
<td>Absent</td>
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<td>1 (14)</td>
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<tr>
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<td>6 (57)</td>
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<td>21 (35)</td>
<td>4 (57)</td>
<td>17 (11)</td>
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</table>

NOTE: All values are expressed as \( n \) (%).

\( a \)Because of limited amount of tumor, we were unable to determine MCPyV status for 2 cases.
PIK3CA. This mutation (R19K) has not been previously reported in the literature and arises from a guanine (G) to adenine (A) change at nucleotide position 1624, resulting in an amino acid substitution of the arginine (R) at codon 19 with a lysine (K). The clinical significance of this variant is currently unknown.

In our cohort, PIK3CA mutations occurred in MCC tumors that presented exclusively in male patients (P = 0.040). Patients with a PIK3CA-mutant MCC presented with stage II–IV disease and the location of their primary tumors included the head and neck (1 case), the lower extremity (1 case), or was undetermined because of disease presentation either in the lymph nodes (2 cases) or as disseminated metastases (1 case; Table 1).

Three of the PIK3CA-mutant MCC cases retrieved for molecular profiling were primary tumors and the remaining 4 tumors were recurrences for which a primary specimen was unavailable for testing. PIK3CA-mutant tumors were significantly larger than their mutation-negative counterparts (median, 8 vs. 2 cm; P < 0.001). In addition, and in contrast to MCPyV-positive tumors, PIK3CA mutations occurred more frequently in tumors with visible necrosis (P = 0.095) and with pleomorphic or spindle shape cell morphology (P = 0.099), but the association between these features and tumor genotype did not reach strict statistical significance due to limited power given the small number of mutant cases (Table 2). These clinicopathologic characteristics were suggestive of a more aggressive tumor behavior and interestingly, with the exception of the H1047L kinase domain mutation, PIK3CA-mutant tumors had a negative MCPyV status. However, we found no association between the presence of a PIK3CA mutation and overall survival of patients with MCCs (Supplementary Table S3, P = 0.628).

The EGFR and the HER2/ERBB2 genes encode for receptor tyrosine kinases that are often overexpressed in human
cancer and that can signal through activation of the PI3K/pAKT pathway. This prompted us to screen our MCC cohort for EGFR and HER2 gene amplification, using FISH analysis. We did not detect EGFR amplification in any of the tumors, but one sample showed HER2 gene amplification. This particular case (MCC-16; Supplementary Table S4) was a local recurrence with a highly unusual histology of very atypical and anaplastic morphologic features, which were most likely secondary to the fact that the primary tumor had been treated with radiation therapy. Unfortunately, the primary tumor was not available for genetic testing, and we could not determine whether HER2 amplification arose after radiation or was present in the original tumor.

For the majority of mutation-positive MCC cases (9 of 11), we were able to obtain matching normal tissue from the same patient and confirm that these were somatic mutations, unique to the tumor (Supplementary Table S4).

**Sensitivity of PIK3CA-mutant MCC cells to PI3K pathway inhibitors**

We had access to 6 primary MCC cell lines, 5 of which (MCC13, MCC26, UISO-mcc1, MKL-1, and MKL-2) had been previously described (18, 24–27). Molecular analysis of these 5 cell lines revealed that none of them harbored PIK3CA mutations and identified 3 that were positive for MCPyV (MKL-1, MKL-2, and UISO-mcc1; Supplementary Table S2). The sixth cell line (MGH-mcc1) was derived from tumor MCC-12, which had been resected from one of the patients in our cohort. Consistent with the molecular profile of the original tumor (Supplementary Table S4), the MGH-mcc1 cell line carried an activating mutation in the helical domain of PIK3CA (Q546K) and was negative for MCPyV.

To evaluate phosphoinositide 3-kinase (PI3K) pathway activation in MCC cell lines, we measured AKT phosphorylation at 2 residues, threonine 308 and serine 473, which are downstream targets of PI3K. AKT phosphorylation could not be detected by immunoblotting in 2 of the 3 MCPyV-positive cell lines, MKL-1 and MKL-2. In contrast, the remaining 4 cell lines yielded robust p-AKT signals, suggesting strong activation of the PI3K/pAKT pathway in a substantial fraction of MCC cell lines (Fig. 4A).

We treated the 6 MCC cell lines with 2 targeted agents under active clinical development: ZSTK474 (32), a PI3K inhibitor, and NVP-BEZ235 (33, 34), a dual inhibitor of PI3K and the mTOR. Exposure to these drugs reduced AKT phosphorylation in all 4 MCC cell lines that exhibited high baseline levels of PI3K signaling (Fig. 4A). In addition, the PIK3CA-mutant cell line (MGH-mcc1) was particularly sensitive to inhibition of the PI3K/pAKT pathway, as evidenced by an increase in apoptosis (Fig. 4B).

**Discussion**

We genotyped 60 patient samples of MCC with the hope of identifying mutations in oncogenes involved in the pathogenesis of this aggressive cancer that could serve as potential targets for small-molecule inhibitors.

The clinical and pathologic characteristics of our cohort of patients with MCC (Table 1) were comparable with those described in previously published studies: the median age at disease presentation was 73 years, the male-to-female ratio was approximately 2:1, immunodeficiency was present in about 13% of patients, and the head and neck region was the most common location of disease at presentation. The median overall survival was lower in our patient population, where we observed approximately 50% overall survival rate at 3 years (Supplementary Table S3) compared with 67% overall survival rate at 3 years or 45% to 65% overall survival rate at 5 years in earlier reports (9, 12, 35). This difference is most likely because of the fact that our institution is a tertiary care center, which serves as a referral center for MCCs. Thus, the majority of patients with
MCCs referred to our service have more advanced and aggressive disease, presenting with a higher disease stage (53%, stage III–IV) than in other reports where the majority of patients present with stage I or II disease (9, 12, 36).

Since its discovery in 2008 (14), MCPyV has been detected in the majority of MCCs, and only occasionally in other cutaneous and hematologic malignancies (15, 37, 38). We detected MCPyV in our cohort at a frequency of 66% (38 of 58 patients), which is well within the wide range of 24% to 100% reported in the literature (35). We observed that MCPyV-positive cases showed a trend toward a better prognosis and prolonged survival, consistent with previous reports (36, 39–41). A similar favorable prognosis for tumors containing viral DNA has also been noted for human papilloma virus–positive oropharyngeal squamous cell carcinomas and for Epstein-Barr virus–positive leiomysosarcomas (42, 43). A possible explanation, at least for MCCs, might be that in the absence of MCPyV, tumorigenesis occurs through more complex or more directly oncogenic genetic abnormalities such as the activation of the PI3K/pAKT pathway. An alternative explanation might be that the immune system of patients whose tumors contain an oncogenic virus, although likely compromised and permissive to a viral infection, might retain some antitumor activity.

Historically, it appears that MCPyV-positive cases are mostly indistinguishable from MCPyV-negative cases, in terms of histologic and clinical features (41). Only a few authors noticed some modest differences between the 2 groups, including lighter, more abundant cytoplasm, and more irregular or polygonal nuclei in virus-negative cases (35, 44) and a higher likelihood of presentation on the extremities (41) for virus-positive cases. In our cohort, most patients with virus-positive MCCs were female (P < 0.001) with lower stage disease at presentation (P = 0.016). In addition, we noticed a significant correlation between the presence of MCPyV and less aggressive histologic features, such as very little or no necrosis (P = 0.004), and the absence of spindle cell or unusual tumor morphology (P = 0.044).

To date, molecular genetic analysis of MCC has been predominantly limited to TP53 revealing mutations in 14 to 28% of cases, mostly through sequencing of the most common hotspots for TP53 mutations on exons 5 to 9 (45, 46). In an effort to better understand the molecular pathogenesis of MCCs, we screened our sample cohort for point mutations in 13 cancer genes (Supplementary Table S1) and detected mutations in 15% of the samples. 5% (3 of 60) of patients had mutations in TP53 and 10% (6 of 60) had mutations in PIK3CA.

Using FISH, we detected HER2 amplification in one unusually pleomorphic, locally recurrent MCC that developed after irradiation of the primary tumor. Because the primary sample was not available for molecular testing, we do not know whether HER2 amplification was a therapy-related event or if, alternatively, it could possibly characterize a yet unrecognized, rare subtype of MCC.

The number of TP53 gene mutations detected in our cohort (5%) was lower than the previously reported frequencies and likely an underestimate, reflecting the fact that single base extension genotyping assays such as SNaPshot, while ideal for oncogenes, are not practical approaches to capture the wide range of mutations typical of tumor suppressors. Interestingly, while other authors identified an inverse correlation between the presence of MCPyV and TP53 mutations, 2 of our 3 TP53-mutant cases (R248P and R273H) were also positive for MCPyV (Supplementary Table S4).

This is the first time, to our knowledge, that activating mutations in PIK3CA, which encodes for the p110α subunit of PI3K, have been detected in MCCs. Activation of the PI3K/pAKT pathway has been observed in a large fraction of human cancers, including colorectal, breast, head and neck, lung, brain, and gynecologic tumors, with mutation frequencies as high as 40% in breast cancers and 75% in head and neck squamous cell carcinomas (47). The majority of mutations so far identified map to 2 hotspots located within the helical and the kinase domains of PI3K. The mutant PIK3CA proteins have higher kinase activity than their wild-type counterparts and can transform chicken embryo fibroblasts and NIH-3T3 cells, supporting the hypothesis that these are “driver” mutations with an active role in tumorigenesis. Therefore, detection of activating PIK3CA mutations in a subset of MCC is highly suggestive of an oncogenic role of the PI3K/pAKT pathway in MCCs.

Using conventional sequencing, we detected one novel mutation in the first exon of PIK3CA, at position c.56G>A (p.R19K). This mutation maps to the adaptor-binding domain located at the extreme N-terminal region of PIK3CA, which is responsible for binding to p85α (48), one of the regulatory subunits of PI3K. Whether this previously unreported mutant could also act as a “driver” is not known, but the R19K substitution was a somatic mutation (absent in normal tissue from the same patient) and involves 2 positively charged and structurally similar amino acid residues at a position that has been conserved through evolution, including in zebrafish, frogs, chicken, and mammals.

Remarkably, some of the clinical and pathologic features of the PIK3CA-mutant MCC are suggestive of a more aggressive tumor behavior, including a significantly larger tumor size (>2 cm), more unusual histopathology, tendency toward a more advanced disease stage at presentation and, with one exception, absence of MCPyV. However, we could not show a statistically significant correlation with worse survival, perhaps due to the relatively small number of PIK3CA-mutant cases. If our survival estimates were found to be reproducible in larger cohorts, it appears that PIK3CA-mutant MCC might have a worse prognosis. Moreover, our observation suggests that a subset of patients with MCCs could potentially benefit from PI3K/pAKT inhibitors, which was corroborated by our cell culture experiments.

We made several attempts to investigate PI3K/pAKT pathway activation in FFPE tumor tissue using immuno-histochemistry with p-AKT antibodies. Unfortunately, we...
were unable to obtain convincing results and decided to
turn instead to the MCC cell lines that were available to us.
Of 6 primary MCC cell lines, 3 were positive for MCPyV and
2 of these showed no activation of the PI3K/pAKT pathway
on immunoblot analysis. The remaining 3 cell lines were
negative for MCPyV and showed a strong activation of the
PI3K/pAKT pathway. These results and the apparent
frequent absence of MCPyV in PIK3CA-mutant MCCs (at least
in our cohort) suggest that genetically distinct subtypes of
MCC may rely on alternative oncogenic mechanisms for
tumorigenesis, which could also explain the different out-
comes of patients with MCPyV-positive versus MCPyV-
negative tumors. There is in fact increasing evidence for an
etiologic role of MCPyV in MCC, such as, the clonal integra-
tion of the virus in the host genome; the constant
expression of small (ST) and large T antigens; the necessity
of the virus for cell growth, survival, and the transformed
phenotype (in cell transformation experiments); and the
almost omnipresent truncation in the 3’ of large T antigens
with loss of replication properties but persistence of the Rh-
binding domain (35).

MCPyV integration and PI3K activation are not mutually
exclusive occurrences, as observed for the MCC-54 primary
tumor sample and for the UISO-mcc1 cell line (Supple-
mental Table S2 and Figure 4), suggesting independent
and possibly cooperative mechanisms of tumorogenesis that
may impact prognosis as well as response to future ther-
apeutic regimens, including potential combinations of anti-
viral and PI3K-targeted agents.

The presence of phosphorylated AKT in 4 of 6 MCC cell
lines suggests the existence of alternative mechanisms for
activation of the PI3K/pAKT pathway, besides point muta-
tions in PIK3CA. Accordingly, inactivating mutations, dele-
tion, or silencing of the PTEN gene, which encodes for a
tumor suppressor that dephosphorylates PIP3 and down-
regulates PI3K/pAKT signaling (49), have been previously
reported in MCCs (26, 50, 51).

Finally, our experiments suggest that PI3K/mTOR inhib-
itors may be an effective treatment approach for the subset
of patients with MCCs with PIK3CA-mutant tumors. These
small-molecule inhibitors have shown promising antitu-
mor activity in the context of PI3K/pAKT pathway activa-
tion, and an expanding number of alternative PI3K/mTOR
inhibitors are undergoing clinical trials for the treatment of
a variety of solid tumors (30, 31, 50–56).

In conclusion, our study shows for the first time that a
subset of MCC cases harbor activating mutations in
PIK3CA, which confer sensitivity to PI3K/mTOR inhibitors
in vitro. Our findings suggest that targeted inhibition of the
PI3K/pAKT pathway could be explored as a possible ther-
apic option for patients with MCCs with PIK3CA-mutant
tumors.

Disclosure of Potential Conflicts of Interest
D. Dias-Santagata and A.J. Iafrate filed a patent application for the
SNIPShot methods used in this study. D. Dias-Santagata, A.J. Iafrate, and
D.R. Borger are consultants for Bio-Reference Laboratories, Inc. J.A. Engel-
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