Activating FGFR2–RAS–BRAF Mutations in Ameloblastoma

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Abstract

**Purpose:** Ameloblastoma is an odontogenic neoplasm whose overall mutational landscape has not been well characterized. We sought to characterize pathogenic mutations in ameloblastoma and their clinical and functional significance with an emphasis on the mitogen-activated protein kinase (MAPK) pathway.

**Experimental Design:** A total of 84 ameloblastomas and 40 non-ameloblastoma odontogenic tumors were evaluated with a combination of BRAF V600E allele-specific PCR, VE1 immunohistochemistry, the Ion AmpliSeq Cancer Hotspot Panel, and Sanger sequencing. Efficacy of a BRAF inhibitor was evaluated in an ameloblastoma-derived cell line.

**Results:** Somatic, activating, and mutually exclusive RAS–BRAF and FGFR2 mutations were identified in 88% of cases. Somatic mutations in SMO, CTNNB1, PIK3CA, and SMARCB1 were also identified. BRAF V600E was the most common mutation, found in 62% of ameloblastomas and in ameloblastic fibromas/fibroductinomas but not in other odontogenic tumors. This mutation was associated with a younger age of onset, whereas BRAF wild-type cases arose more frequently in the maxilla and showed earlier recurrences. One hundred percent concordance was observed between VE1 immunohistochemistry and molecular detection of BRAF V600E mutations. Ameloblastoma cells demonstrated constitutive MAPK pathway activation in vitro. Proliferation and MAPK activation were potently inhibited by the BRAF inhibitor vemurafenib.

**Conclusions:** Our findings suggest that activating FGFR2–RAS–BRAF mutations play a critical role in the pathogenesis of most cases of ameloblastoma. Somatic mutations in SMO, CTNNB1, PIK3CA, and SMARCB1 may function as secondary mutations. BRAF V600E mutations have both diagnostic and prognostic implications. In vitro response of ameloblastoma to a BRAF inhibitor suggests a potential role for targeted therapy.

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Introduction

Ameloblastoma is a locally invasive odontogenic neoplasm arising in the mandible or maxilla. Most ameloblastomas are slow-growing neoplasms without metastatic potential, but surgical resection often results in significant facial deformity and recurrences are common (1). Although the mutational landscape of ameloblastoma has not been well characterized, there is mounting evidence for the activation of the mitogen-activated protein kinase (MAPK) pathway playing a prominent role in the pathogenesis of ameloblastoma. Several studies have demonstrated activation of components of the MAPK pathway in an ameloblastoma cell line (AM-1) under various circumstances, including stimulation with tumor necrosis factor α (2) and fibroblast growth factors 7 and 10 (3). In addition, transgenic mice expressing v-Ha-Ras under the ζ-globin promoter develop odontogenic tumors resembling ameloblastoma (4). We recently identified a case of metastatic ameloblastoma with a BRAF V600E mutation. This same mutation was recently reported in 15 of 24 benign ameloblastomas (5). Single cases of ameloblastomas with KRAS (6) and CTNNB1 (7) mutations have also been described. However, a comprehensive evaluation of the MAPK pathway or other cancer-associated mutations has not been performed. In this study, we sought to determine the overall frequency of MAPK and other mutations in a large cohort of ameloblastomas, to investigate the functional and clinical significance of these mutations, and to establish the specificity of these mutations compared with other odontogenic tumors.

Materials and Methods

**Sample selection**

With institutional review board approval at the University of Michigan, medical records were queried for samples from patients diagnosed with ameloblastoma over the preceding 15 years. A schematic of how available cases...
Translational Relevance

The pathogenesis of ameloblastoma is poorly understood. Here, we show that activating FGFR2–RAS–BRAF mutations are present in 88% of ameloblastomas, with BRAF V600E being the most common (62%). Among odontogenic tumors, BRAF mutations are specific for those with ameloblastic epithelium, suggesting a potential role as a diagnostic marker. One hundred percent concordance was observed between VE1 immunohistochemistry and molecular detection of BRAF mutations. We show that BRAF V600E is associated with a younger age of onset, whereas BRAF V600E-negative cases arise more frequently in the maxilla. The absence of a BRAF mutation is also an independent risk factor for early recurrence. Somatic mutations in SMO, CTNNB1, PIK3CA, and SMARCBI co-occur with FGFR2–RAS–BRAF mutations, and thus may function as secondary mutations. In vitro proliferation of ameloblastoma cells and MAPK pathway activation are potentially inhibited by the BRAF inhibitor vemurafenib, suggesting a potential role for targeted therapy in the treatment of ameloblastoma.

were evaluated is depicted in Fig. 1A. Forty cases of non-ameoblastoma odontogenic tumors were also accrued for evaluation of BRAF V600E status using allele-specific PCR. These included 1 ameloblastic carcinoma, 2 ameloblastic fibromas, 1 ameloblastic fibrodenitma, 1 odontomaameloblastoma, 5 clear cell odontogenic carcinomas, 2 adenomatoide odontogenic tumors, 19 keratocystic odontogenic tumors, 2 calcifying cystic odontogenic tumors, 1 calcifying epithelial odontogenic tumor, 1 odontogenic fibroma, and 5 odontogenic myxomas. All diagnoses were confirmed by an experienced head and neck pathologist (J.B. McHugh).

DNA isolation
DNA was extracted from available non-decalcified, formalin-fixed paraffin-embedded samples in areas containing at least 30% tumor nuclei using the Pinpoint Slide DNA Isolation System (Zymo Research) according to the manufacturer's instructions.

Cell line
The AM-1 ameloblastoma cell line was obtained from the laboratory of Dr. Hidemitsu Harada at Iwate Medical University (8). SK-MEL-28 (BRAF V600E–positive) and MCF7 (BRAF wild-type) cells were obtained from the American Type Culture Collection. AM-1 cells were maintained in Keratinocyte-SFM (GIBCO) supplemented with bovine pituitary extract. SK-MEL-28 and MCF7 cell lines were maintained in DMEM medium (Hyclone) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone) and 5% CO2. Cell line purity and identity was confirmed by evaluation of microscopic morphology and BRAF exon 15 sequencing.

BRAF V600E allele-specific PCR
Allele-specific PCR screening for BRAF V600E was performed as described by Brown and colleagues (9).

Ion AmpliSeq Cancer Hotspot Panel
Sequencing libraries were generated using Life Technologies’ Ion AmpliSeq Cancer Hotspot Panel v2. This panel consists of 207 amplicons covering mutation hotspots in 50 genes, including KRAS, HRAS, NRAS, and BRAF. Approximately 10 ng of starting DNA from each sample block was amplified. Each Library was barcoded (IonXpress Barcode Kit, Life Technologies) and equalized (Ion Library Equalizer Kit) to a final concentration of approximately 100 pmol/L. Emulsification PCR using 8 to 16 combined barcoded libraries was performed using the One Touch 2 instrument. Template-positive Ion Sphere particles were then enriched using the One Touch ES instrument per the manufacturer’s recommendations. Sequencing was performed on a 318v2 chip on the Ion Torrent PGM following the recommended protocol. Reads were aligned to hg19 and variants were called using the Torrent Suite 3.6.2 and Ion Reporter 4.0. Variants were assessed using the Broad Institute’s Integrated Genomics Viewer (IGV 2.3).

Mutation validation
Candidate somatic variants from the Ion AmpliSeq Cancer Hotspot Panel were defined as those in regions with a depth greater than \( \times 100 \) and a variant frequency greater than 10%. Synonymous variants and variants registered in dbSNP131 were excluded. Candidate somatic variants were evaluated using bidirectional Sanger sequencing with nested sequencing primers. Tumor and germline DNA (when available) were compared. Variants present in tumor DNA and not in germline DNA were categorized as somatic variants.

Immunohistochemistry
Two tissue microarrays (TMA) were constructed with 1 mm cores in triplicate from formalin-fixed paraffin-embedded (FFPE) tissue blocks. One TMA was prepared from the 50 non-decalcified cases suitable for molecular evaluation. Decalcified and non-decalcified cores from 10 BRAF V600E–positive cases were included in this array in order to assess the effect of decalcification on immunohistochemistry. A separate microarray was constructed from 34 cases of ameloblastomas not suitable for molecular evaluation because of decalcification. For cases in which tumor tissue was not present within the array, whole sections were evaluated. Four-μm-thick sections were cut and processed for immunohistochemistry using an antibody against V600E-mutated BRAF protein (clone VE1, Spring Bioscience, dilution 1:40). Immunoreactivity was independently scored by 2 pathologists. Positive cases were defined as those showing clear immunoreactivity in the majority of neoplastic cells (>50%). CTNNB1 (β-catenin, clone 14, Cell
Marque, predilute) and SMARCB1 (INI-1, clone MRQ-27; Cell Marque, predilute) immunohistochemistry was performed in cases with CTNNB1 and SMARCB1 mutations, respectively. For comparison, 6 CTNNB1/SMARCB1 wild-type cases (3 BRAF mutated and 3 BRAF wild type) were also evaluated with these stains. See Supplementary Methods for staining procedures.

Western blotting
Cell lines were incubated in vehicle (DMSO) or vemurafenib (PLX4032) at 150, 500, or 1,000 nmol/L for 2 or 6 hours. Pellets were lysed in RIPA buffer (Thermo Scientific). Western blotting analyses were performed by the standard protocol using assorted primary antibodies: anti-pERK (E-4, Santa Cruz Biotechnology, 1:500 dilution), anti-ERK (C-16, Santa Cruz Biotechnology, 1:1,000 dilution), anti-p-MEK 1/2 (Cell Signaling Technology; 1:1,000 dilution), anti-MEK 1/2 (Cell Signaling Technology; 1:1,000 dilution), and anti-GAPDH (MAB374, Millipore; 1:1,000 dilution).

Proliferation assay
Cell lines were incubated in vehicle (DMSO) or vemurafenib at 10, 50, 100, 200, 500, 1,000, or 10,000 nmol/L. Cell growth was determined at 24, 72, 96, 144, 192, and 216 hours in triplicate by measurement of metabolic cleavage of tetrazolium salt into formazan using Cell Proliferation Reagent WST-1 (Roche Diagnostics) according to the manufacturer’s instructions. The IC50 was determined from the regression of a plot of the logarithm of the concentration versus percent inhibition at the time point of maximal inhibition. Because of the slow growth and long doubling time of AM-1, the IC50 was determined at 216 hours whereas the IC50 of SK-MEL-28 was determined at 96 hours.

Statistical analyses
Clinical variables, including age at diagnosis, tumor location (mandible vs. maxilla), tumor size, and treatment procedure (resection vs. enucleation), were evaluated for a potential association with BRAF mutation status. Continuous variables were expressed as mean with standard deviation and association with BRAF mutation status was evaluated using a 2-sample t test. Categorical variables were expressed as a percentage (frequency) and association with BRAF mutation status was evaluated using the \( \chi^2 \) test. Recurrence-free survival (RFS) was calculated by the Kaplan–Meier method, and compared using the log-rank test. Univariate Cox proportional hazards regression analysis was used to evaluate BRAF mutation and each clinical variable on the survival outcome, and the multivariate Cox regression was used to evaluate the effect of BRAF mutation status on survival after adjusting for age, tumor location and treatment procedure. All analyses were performed using SAS (version 9.3, SAS Institute). A \( P \) value of <0.05 was considered significant.

Results
BRAF V600E mutations in ameloblastoma
A BRAF V600E mutation was initially identified within our laboratory from a 29-year-old female with metastatic...
ameloblastoma while being evaluated for clinical trials. This mutation was identified using an allele-specific PCR test for BRAF V600E and confirmed with Sanger Sequencing (Fig. 1B and C). To determine the prevalence of BRAF V600E in ameloblastoma, 50 cases with non-decalcified tissue were selected and evaluated using the BRAF V600E allele–specific PCR test. BRAF V600E mutations were identified in 31 of 50 cases (62%).

We next used VE1 immunohistochemistry to establish the utility of this stain in the context of ameloblastoma and to broaden the cohort to include cases with tissue not amenable to DNA-based mutation screening because of decalcification. Immunohistochemistry was first performed on 50 cases previously evaluated by BRAF V600E allele–specific PCR (44 within a tissue microarray and 6 within whole tissue sections). 100% concordance for BRAF V600E mutation status was observed between molecular and immunohistochemistry results for the 50 cases evaluated by both techniques. Decalcified cores from 10 BRAF V600E–positive cases included in this microarray showed staining of similar intensity to paired non-decalcified cores. Neoplastic epithelium showed staining that was consistently cytoplasmic and ranged from weak to moderate in intensity, typically strongest in the basal cells (Fig. 1D). Tumor cells with squamous morphology frequently displayed weaker staining. No staining was observed in non-neoplastic tissue.

A separate tissue microarray comprised of 34 cases of ameloblastomas not suitable for molecular evaluation because of decalcification was evaluated using VE1 immunohistochemistry. Evidence of a BRAF V600E mutation was observed in a similar proportion of cases as those evaluated by molecular techniques—23/34 (65.7%) compared with 31/50 (62%). 100% concordance was observed between 2 independent scorers.

**Additional FGFR2–RAS–BRAF mutations**

To screen for additional MAPK pathway mutations in ameloblastomas, we evaluated 50 cases using the Ion AmpliSeq Cancer Hotspot panel. No BRAF mutations other than V600E were identified. Activating, somatically acquired RAS mutations were mutually exclusive with BRAF mutations and were found in 10 of 19 BRAF wild-type cases (53%; Fig. 2A and B; Supplementary Fig. S1 and Table S1). RAS mutations included 4 KRAS mutations (8%; G12R), 3 NRAS mutations (6%; Q61R and Q61K), and 3 HRAS mutations (6%; G12S, Q61R, and Q61K). The allele frequency is greater than 50% in some cases, suggesting possible homozygosity or aneuploidy. Mutually exclusive FGFR2 mutations were also identified in 3 cases (6%; C382R and V395D). All 3 cases demonstrated allele frequencies greater than 50% consistent with homozygosity or aneuploidy. Together BRAF, RAS, and FGFR2 mutations were present in 44 of 50 ameloblastomas (88%). All mutations were confirmed by Sanger sequencing. The somatic nature of these mutations was confirmed by sequencing matched normal DNA in 5 cases with BRAF mutations; 3 cases with KRAS mutations; and all cases with NRAS, HRAS, and FGFR2 mutations.

**Other mutations**

The Ion AmpliSeq Cancer Hotspot panel also identified several recurrent somatic mutations not directly involved in the MAPK pathway (Supplementary Fig. S1 and Table S1). These included SMO L412F (4), W535L (3) and G416E (1); CTNNB1 S33P (1), S45P (1); PIK3CA E542K (1), E545K (1), H1047R (1); and SMARCB1 R377H (3). All of these missense mutations were previously described in other neoplasms except SMO G416E. Sanger sequencing of tumor with matched normal tissue confirmed the somatic nature of each of these mutations. These mutations co-occurred with FGFR2–RAS–BRAF mutations and, in some cases, with each other (Fig. 2B). CTNNB1 (β-catenin) immunohistochemistry demonstrated membranous staining without any difference in staining intensity or pattern in CTNNB1-mutated and CTNNB1 wild-type tumors (Supplementary Fig. S2A). Likewise, SMARCB1 (INI-1) staining did not differ between SMARCB1 mutated and SMARCB1 wild-type ameloblastomas (Supplementary Fig. S2B). Variants found in both tumor and germline DNA were considered to be benign polymorphisms. All somatic and germline variants are listed in Supplementary Table S1.
Other odontogenic tumors

To determine the specificity of the BRAF V600E mutation in the context of odontogenic tumors, we evaluated 40 non-ameloblastoma odontogenic tumors using BRAF V600E allele–specific PCR. BRAF V600E mutations were identified in 1/1 ameloblastic fibrodentinomas and 2/2 ameloblastic fibromas. All other odontogenic tumors were negative for BRAF V600E (Supplementary Table S2).

Clinical/genotypic association

Clinical and genotypic information for each case is shown in Supplementary Table S1. BRAF V600E mutations were correlated with a younger age of diagnosis for all 84 cases in this study (mean age 34.5 vs. 53.6; \( P < 0.0001 \); Table 1) and for the initial 50 non-decalcified cases of ameloblastoma evaluated by molecular techniques (mean age 33.0 vs. 53.4; \( P = 0.0007 \)). Cases with BRAF mutations were also less likely to arise in the maxilla—rather than mandible—for all cases (5.6% vs. 43.3%; \( P < 0.0001 \}; Table 1) and for those evaluated with molecular techniques (9.7% vs. 68.4%; \( P < 0.0001 \)). No association was found between BRAF mutation status and tumor size or treatment procedure performed. In univariate analyses, earlier recurrences were seen in BRAF V600E wild-type tumors (log-rank \( P = 0.0072 \}; Fig. 3A), in maxillary tumors (log-rank = 0.035; Fig. 3C), and in patients treated with enucleation rather than resection (log-rank = 0.017; Fig. 3D). In a multivariate analysis (Table 2), only BRAF status (\( P = 0.0455 \)) and treatment procedure (\( P = 0.0207 \)) showed an independent, statistically significant association with recurrence-free survival.

In vitro inhibition of BRAF

To determine the functional significance of BRAF V600E mutations in ameloblastomas and to assess the potential utility of therapeutic agents targeting this mutation, we evaluated the in vitro effect of vemurafenib on MAPK
pathway activation and proliferation of the ameloblastoma cell line AM-1. A melanoma cell line known to harbor a BRAF V600E mutation, SK-MEL-28, was used as a positive control whereas a breast cancer cell line without any known MAPK pathway mutations, MCF7, was included as a negative control. Sanger sequencing of BRAF exon 15 demonstrated a BRAF V600E mutation in AM-1, a homozygous BRAF V600E mutation in SK-MEL-28, and no mutations in MCF7 (Fig. 4A). Both AM-1 and SK-MEL-28 demonstrated high levels of phospho-ERK and phospho-MEK relative to MCF7. This activation was inhibited by vemurafenib (a BRAF small molecule inhibitor) in a dose- and time-dependent manner (Fig. 4B). Vemurafenib inhibited the proliferation of AM-1 and SK-MEL-28 without affecting the proliferation of MCF7 (Fig. 4C). The IC50 for AM-1 and SK-MEL-28 were 119 and 476 nmol/L, respectively (Fig. 4D). The IC50 for MCF7 was not reached.

Discussion

Ameloblastoma is a locally invasive odontogenic neoplasm whose pathogenesis is poorly understood. Several studies have demonstrated activation of the MAPK pathway in ameloblastomas cells under various circumstances (2, 3). In addition, transgenic mice with v-Ha-Ras have been shown to develop odontogenic tumors resembling ameloblastoma (4). We recently identified a BRAF V600E mutation in a case of metastatic ameloblastoma. During the preparation of this manuscript, 2 studies reported the same mutation in benign ameloblastomas. Kurppa and colleagues reported BRAF V600E mutations in 15 of 24 cases but did not evaluate the functional and clinical significance of this mutation, the specificity of this mutation for ameloblastoma, or the effect of BRAF inhibitor therapy (5). Sweeney and colleagues also reported BRAF mutations as well as KRAS, FGFR2, and SMO mutations in a cohort of 28 cases (10). This study did not identify HRAS, NRAS, SMARCB1, CTNNB1, or PIK3CA mutations and included an insufficient number of cases to establish a significant association between genotype and prognosis. In this study, we evaluated a total of 84 ameloblastoma cases using a combination of mutation screening techniques and identified MAPK pathway (BRAF, KRAS, NRAS, HRAS) and FGFR2 mutations in 88% of cases as well as several non-MAPK mutations, including SMO, SMARCB1, CTNNB1, and PIK3CA. We also evaluated the functional and clinical significance of the most common mutation in ameloblastoma—BRAF V600E—including in vitro response to targeted therapy. Finally, we evaluated other non-ameloblastoma odontogenic tumors to establish the specificity of BRAF V600E.

Among ameloblastomas evaluated with molecular techniques, somatic mutations were identified in 92% of cases. Most cases with multiple mutations showed similar allele frequencies except for 2 cases in which the frequency of

### Table 1. Clinical characteristics of patient with BRAF V600E–mutated and wild-type ameloblastoma

<table>
<thead>
<tr>
<th>Continuous variables</th>
<th>BRAF V600E (n = 54)</th>
<th>BRAF wild-type (n = 30)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at diagnosis (years)</td>
<td>34.5 (17.8) 8–81</td>
<td>53.6 (19.4) 13–84</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Tumor size (cm)</td>
<td>4.3 (2.6) 0.5–11.0</td>
<td>3.5 (1.5) 0.5–6.5</td>
<td>0.1143</td>
</tr>
<tr>
<td>Categorical variables</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Location</td>
<td>Number Percentage</td>
<td>Number Percentage</td>
<td>P</td>
</tr>
<tr>
<td>Mandible</td>
<td>51 (94.4%)</td>
<td>17 (56.7%)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Maxilla</td>
<td>3 (5.6%)</td>
<td>13 (43.3%)</td>
<td></td>
</tr>
<tr>
<td>Treatment procedure</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enucleation</td>
<td>6 (12.5%)</td>
<td>4 (13.8%)</td>
<td>1.00</td>
</tr>
<tr>
<td>Resection</td>
<td>42 (87.5%)</td>
<td>25 (86.2%)</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviation: SD, standard deviation.

### Table 2. Multivariate cox analysis of recurrence-free survival

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Parameter estimate</th>
<th>P value</th>
<th>Hazard ratio (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype (BRAF V600E vs. wild-type)</td>
<td>−1.79533</td>
<td>0.0455</td>
<td>0.166 (0.029–0.965)</td>
</tr>
<tr>
<td>Age at diagnosis</td>
<td>−0.01152</td>
<td>0.525</td>
<td>0.988 (0.954–1.024)</td>
</tr>
<tr>
<td>Tumor location (mandible vs. maxilla)</td>
<td>−0.63145</td>
<td>0.3294</td>
<td>0.532 (0.15–1.892)</td>
</tr>
<tr>
<td>Treatment procedure (enucleation vs. resection)</td>
<td>1.76921</td>
<td>0.0207</td>
<td>5.866 (1.311–26.249)</td>
</tr>
</tbody>
</table>

Abbreviation: CI, confidence interval.
FGFR2 mutations were approximately double that of SMO mutations and 1 case in which the frequency a SMARCB1 mutation was approximately double that of an HRAS mutation. Another 2 cases showed RAS mutations at allele frequencies greater than 50%. These differences likely reflect homozygous mutations but could also result from aneuploidy, amplification, etc.

BRAF V600E was the most common mutation found in 62% of cases evaluated by molecular techniques and 66% of cases evaluated by VE1 immunohistochemistry. We confirmed this mutation to be somatic by documenting the absence of this mutation in germline DNA. Kurppa and colleagues (5) recently identified this mutation in a similar proportion of cases—63% (15 of 24 cases), whereas Sweeney and colleagues (10) reported BRAF mutations at a lower frequency—46% (13/28). BRAF is a serine–threonine kinase within the MAPK pathway. The V600E mutation results in constitutive activation of this enzyme and downstream MEK and ERK signaling, enhancing cell proliferation, survival, and ultimately neoplastic transformation (11). This mutation is present in numerous neoplasms, including melanoma (12), hairy cell leukemia (13), papillary thyroid carcinoma (14), Langerhans cell histiocytosis (15), and colorectal cancer (16).

Forty non-ameloblastoma odontogenic tumors were evaluated for the BRAF V600E mutation using allele-specific PCR. The BRAF V600E mutation was identified in 1 of 1 ameloblastic fibrodentinoma and 2 of 2 ameloblastic fibromas, suggesting these neoplasms may represent histologic variants of ameloblastoma with a similar pathogenesis. All other odontogenic tumors evaluated were negative for BRAF V600E. These findings suggest ameloblastic tumors are a distinct group of odontogenic tumors with characteristic genetic abnormalities. These findings also implicate the BRAF V600E mutation as a potential diagnostic marker. In this study, 100% concordance was observed between VE1 immunohistochemistry and molecular detection of BRAF V600E mutations, demonstrating that both techniques may be useful in the diagnosis of ameloblastoma.

In this study, BRAF V600E mutations were more frequently observed in younger patients and less frequently observed in tumors arising in the maxilla. These findings are analogous to BRAF V600E mutations in melanoma, which also occur in younger patients and have a different anatomic distribution compared with NRAS and other mutations (17–20). In melanoma, different anatomic distributions are thought to result from differences in ultraviolet light...
exposure. It is unclear why the anatomic distribution differs between BRAF V600E and BRAF wild-type ameloblastomas. BRAF mutation status was also a predictor of recurrence-free survival. In univariate analyses, earlier recurrences were seen in BRAF wild-type tumors. Earlier recurrences were also observed in those treated with enucleation rather than resection as previously reported (21) and in tumors arising in the maxilla. The association between the BRAF mutation status and recurrences may be explained in part by the more frequent localization of BRAF wild-type tumors in the maxilla. However, in a multivariate analysis that included BRAF status, age at diagnosis, tumor location, and treatment procedure, both BRAF status (P = 0.0455) and treatment procedure (P = 0.0207) were independent, statistically significant predictors of recurrence. This finding implicates BRAF V600E as a prognostic marker.

The BRAF protein is normally activated by the G-protein RAS. KRAS, NRAS, and HRAS are oncogenes commonly mutated in cancer (22). In this study, mutations in KRAS, NRAS, and HRAS were present in 53% of BRAF V600E negative cases of ameloblastoma. All RAS mutations occurred at sites commonly mutated in other neoplasms—codons 12 and 61. Fibroblast growth factor receptor 2 (FGFR2) is one of several receptors that activate RAS. Activating FGFR2 mutations occur in 10% of endometrial carcinomas and are mutually exclusive with KRAS mutations (23). These mutations result in constitutive MAPK pathway activation that is abrogated by treatment with FGFR inhibitors (24). In this study, somatic FGFR2 mutations (C382R and V395D) were found in 6% of ameloblastoma cases. Both mutations affect the transmembrane domain of the receptor and have been described in endometrial carcinoma (23–26). The C382R mutation has been shown to result in constitutive activation (27) and to transform 3T3 cell in vitro (25, 27). Furthermore, treatment of ameloblastoma cells with FGFR2 ligands FGF7 and FGF10 results in cell proliferation and MAPK pathway activation, an effect that can be blocked by MEK inhibition (3). In this study, RAS, BRAF, and FGFR2 mutations were found to be mutually exclusive with one another and together were found in 88% of cases. This pattern suggests each of these mutations likely represents a critical mutation affecting a common pathway and occurring early in the pathogenesis of ameloblastoma.

Several mutations were identified within genes not involved in the MAPK pathway. These included SMO, CTNNB1, PIK3CA, and SMARCB1. All mutations were confirmed to be somatically acquired. In contrast to FGFR2–RAS–BRAF mutations, these were present in a lower proportion of cases (16%, 4%, 6%, and 6%, respectively), and were not mutually exclusive with FGFR2–RAS–BRAF mutations or with one another, suggesting they may represent secondary mutations occurring later in the pathogenesis of ameloblastoma.

In this study, SMO was the most commonly mutated gene outside of RAS–BRAF with mutations present in 16% of cases overall. These mutations occurred both in isolation (1), and in cases with concomitant RAS (4), FGFR2 (2), and BRAF (1) mutations. Although W535L and L1412F have been described in basal cell carcinoma (28, 29) and meningioma (30, 31) and have been shown to be activating (20, 32), G416E represents a novel mutation. The Smoothen (SMO) protein is a nonclassical G-protein–coupled receptor normally repressed by patched (PTCH1) in its resting state (33). Although SMO mutations have never been described in association with ameloblastoma, polymorphism and deleterious germline mutations within PTCH1 have been shown to affect the risk of ameloblastoma (34, 35).

Two mutations, S33P and S45P, were identified in the CTNNB1 gene, which encodes the β-catenin protein—a key downstream component of the Wnt signaling pathway. One mutation was present in association with both BRAF and PIK3CA mutations. A single case of ameloblastoma with CTNNB1 S45P was previously reported in the literature (7) whereas S33P has never been described in ameloblastoma. Both of these mutations involve serine residues normally phosphorylated before ubiquitination and both mutations have been described in other neoplasms, including desmoid tumors (36) and hepatocellular carcinoma (37). No difference was observed in the CTNNB1 (β-catenin) staining pattern or intensity for CTNNB1-mutated and CTNNB1 wild-type ameloblastomas. Of note, frequently mutated sites in the APC gene are included within the Ion AmpliSeq Cancer Hotspot panel, but no APC mutations were identified in this study.

A SMARCB1 R377H mutation was identified in 3 cases of ameloblastomas, 2 that also carried a BRAF V600E mutation and 1 that also had a HRAS mutation. This mutation has been described in both meningioma (44) and gastric carcinoma (45). In ameloblastoma, this missense mutation did not alter (SMARCB1) INI-1 staining. Of note, one patient with this mutation suffered from very locally aggressive disease with invasion of both orbits and the right temporal lobe, ultimately resulting in the patient’s death. This same mutation was found in a case of ameloblastic carcinoma. It is possible this represents a secondary mutation within ameloblastic tumors that results in a more aggressive behavior.

Several small molecule inhibitors targeting BRAF and MEK are FDA approved or in clinical trials for the treatment of neoplasms with activating MAPK pathway mutations, principally BRAF V600E-mutated melanoma. We evaluated the in vitro efficacy of the BRAF inhibitor vemurafenib using the ameloblastoma cell line AM-1 that harbors a BRAF V600E mutation. Both AM-1 and a melanoma cell line with a BRAF V600E mutation (SK-MEL-28) showed evidence of MAPK pathway activation with high levels of phospho-ERK and phospho-MEK relative to MCF7 cells. This activation was inhibited by
vemurafenib in a dose- and time-dependent manner. Vemurafenib also inhibited the proliferation of AM-1 and SK-MEL-28 without affecting the proliferation of MC7. Interestingly, vemurafenib seems to have a more potent effect in AM-1 cells than in the melanoma cell line with a homozygous BRAF V600E mutation as the I_{50} for AM-1 was 119 nmol/L compared with 476 nmol/L for SK-MEL-28. These findings suggest a potential role for BRAF inhibitors in ameloblastoma treatment. Although ameloblastoma is typically treated surgically, surgical resection often results in significant facial deformity and recurrences are common. In addition, pharmacologic treatment may be useful in metastatic and locally aggressive cases and in patients who are poor surgical candidates.

Overall, our findings suggest somatic FGFR2–RAS–BRAF mutations are critical mutations in the pathogenesis of most cases of ameloblastoma. Somatic mutations in SMO, CTNNB1, PIK3CA, and SMARCB1 may function as secondary mutations. The most common mutation—BRAF V600E was not found in odontogenic neoplasms without ameloblastic epithelium, suggesting a potential role as a diagnostic marker. This mutation also displayed clinical and prognostic significance. BRAF V600E was associated with a younger age of onset whereas BRAF wild-type cases arose more frequently in the maxilla and showed earlier recurrences. Proliferation of ameloblastoma cells and MAPK pathway activation are potently inhibited by the BRAF inhibitor vemurafenib, suggesting a potential role for targeted therapy in the treatment of ameloblastoma.

Disclosure of Potential Conflicts of Interest
M.S. Lim is a consultant/advisory board member for Janssen Pharmaceutical and Rand Corp. No potential conflicts of interest were disclosed by the other authors.

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