Activating \textit{FGFR2-RAS-BRAF} Mutations in Ameloblastoma

Noah A. Brown\textsuperscript{1}, Delphine Rolland\textsuperscript{1}, Jonathan B. McHugh\textsuperscript{1}, Helmut C. Weigelin\textsuperscript{1}, Lili Zhao\textsuperscript{2}, Megan S. Lim\textsuperscript{1}, Kojo S.J. Elenitoba-Johnson\textsuperscript{1}, Bryan L. Betz\textsuperscript{1}

\textsuperscript{1}Department of Pathology, University of Michigan, Ann Arbor, Michigan, USA
\textsuperscript{2}Department of Biostatistics, University of Michigan, Ann Arbor, Michigan, USA

\textbf{Correspondence:}

Bryan L. Betz, PhD
Department of Pathology
University of Michigan
Traverwood 2, Suite C
2910 Huron Parkway
Ann Arbor, Michigan 48105-3450
Telephone: 734-764-0569
Fax: 734-764-4102
E-mail: bbetz@med.umich.edu

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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 \textit{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/fibrodentinomas but not in other odontogenic tumors. This mutation was associated with a younger age of onset while BRAF wild-type cases arose more frequently in the maxilla and showed earlier recurrences. 100% concordance was observed between VE1 immunohistochemistry and molecular detection of BRAF V600E mutations. Ameloblastoma cells demonstrated constitutive MAPK pathway activation \textit{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. \textit{In vitro} response of ameloblastoma to a BRAF inhibitor suggests a potential role for targeted therapy.

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. 100% 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 while 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 SMARCB1 co-occur with FGFR2-RAS-BRAF mutations and thus may function as secondary mutations. \textit{In vitro} 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.
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). While 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 alpha (2) and fibroblast growth factors 7 and 10 (3). In addition, transgenic mice expressing v-Ha-Ras under the zeta-globin promoter develop odontogenic tumors resembling ameloblastoma (4). We recently identified a case of metastatic ameloblastoma with a \textit{BRAF} V600E mutation. This same mutation was recently reported in 15 of 24 benign ameloblastomas (5). Single cases of ameloblastomas with \textit{KRAS} (6) and \textit{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 to 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 fifteen years. A schematic of how available cases were evaluated is depicted in Figure 1A. Forty cases of non-ameloblastoma odontogenic tumors were also accrued for evaluation of \textit{BRAF} V600E status using allele-specific PCR. These included one ameloblastic carcinoma, two ameloblastic fibromas, one ameloblastic fibrodentinoma,
one odontoameloblastoma, five clear cell odontogenic carcinomas, two adenomatoid odontogenic tumors, nineteen keratocystic odontogenic tumors, two calcifying cystic odontogenic tumors, one calcifying epithelial odontogenic tumor, one odontogenic fibroma and five odontogenic myxomas. All diagnoses were confirmed by an experienced head and neck pathologist (JBM).

**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 Lines**

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). Cultures were kept at 37°C in a humidified atmosphere with 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 et al (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 pM. Emulsification PCR using 8-16 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 100X 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 1mm 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 due to decalcification. For cases in which tumor tissue was not present within the array, whole sections were evaluated. 4 micron-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 two pathologists. Positive cases were defined as those showing clear immunoreactivity in the majority of neoplastic cells (>50%). CTNNB1 (Beta-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 Supplemental Methods for staining procedures.

**Western blotting**

Cell lines were incubated in vehicle (DMSO) or vemurafenib (PLX4032) at 150nM, 500nM or 1,000nM for two hours or six 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 10nM, 50nM, 100nM, 200nM, 500nM, 1,000nM or 10,000nM. 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 IC$_{50}$ was determined from the regression of a plot of the logarithm of the concentration versus percent inhibition at the time point of maximal inhibition. Due to the slow growth and long doubling time of AM-1, the IC$_{50}$ was determined at 216 hours while the IC$_{50}$ of SK-MEL-28 was determined at 96 hours.

**Statistical Analyses**
Clinical variables including age at diagnosis, tumor location (mandible versus maxilla), tumor size, and treatment procedure (resection versus 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 two-sample t test. Categorical variables were expressed as a percentage (frequency) and association with \( BRAF \) mutation status was evaluated using Chi-Square 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, Cary, NC). A p-value of <0.05 was considered significant.

RESULTS

\textit{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-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 employed 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 due to 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 due to 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 to 31/50 (62%). 100% concordance was observed between two independent scorers.

**Additional FGFR2-RAS-BRAF Mutations**

In order 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-B; Supplemental Fig. S1; Supplemental 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 frequencies 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 three cases demonstrated allele frequencies greater than 50% consistent with homozygosity or aneuploidy. Together *BRAF*, *RAS* and *FGFR2* mutations were present in 44/50 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 (Supplemental Fig. S1; Supplemental 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 each of these mutations. These mutations co-occurred with FGFR2-RAS-BRAF 
mutations and, in some cases, with each other (Fig. 2B). CTNNB1 (Beta-catenin) immunohistochemistry 
demonstrated membranous staining without any difference in staining intensity or pattern in CTNNB1 
mutated and CTNNB1 wild-type tumors (Supplemental Fig. S2A). Likewise, SMARCB1 (INI-1) staining 
did not differ between SMARCB1 mutated and SMARCB1 wild-type ameloblastomas (Supplemental Fib. 
S2B). Variants found in both tumor and germline DNA were considered to be benign polymorphisms. 
All somatic and germline variants are listed in Supplemental Table S1.

Other Odontogenic Tumors

To determine the specificity of the BRAF V600E mutation in the context of odontogenic tumors, we 
evaluated forty 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 (Supplemental Table S2).

Clinical/Genotypic Association

Clinical and genotypic information for each case is shown in Supplemental Table S1. BRAF V600E 
mutations were correlated with a younger age of diagnosis for all 84 cases in this study (mean age 34.5
versus 53.6; p<0.0001; Fig. 3A) and for the initial 50 non-decalcified cases of ameloblastoma evaluated by molecular techniques (mean age 33.0 versus 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% versus 43.3%; p<0.0001) and for those evaluated with molecular techniques (9.7% versus 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=0.0072; Fig. 3B), 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 while 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 119nM and 476nM, 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, two studies reported the same mutation in benign ameloblastomas. Kurppa et al. 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 et al. 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 two cases in which the frequency of FGFR2 mutations were approximately double that of SMO mutations and one case in which the frequency a SMARCB1 mutation was approximately double that of an HRAS mutation. Another two 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 et al. (5) recently identified this mutation in a similar proportion of cases – 63% (15 of 24 cases), while Sweeney et al. (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 1/1 ameloblastic fibrodentinoma and 2/2 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. While W535L and L412F 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 Smoothened ($SMO$) protein is a non-classical G-protein-coupled receptor normally repressed by patched ($PTCH1$) in its resting state (33). While $SMO$ 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 beta-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) while S33P has never been described in ameloblastoma. Both of these mutations involve serine residues normally phosphorylated prior to ubiquitination and both mutations have been described in other neoplasms including desmoid tumors (36) and hepatocellular carcinoma (37). No difference was observed in the $CTNNB1$ (beta-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.

PIK3CA mutations – E542K, E545K, H1047R – were identified in three cases, one with both a BRAF and a CTNNB1 mutation. PIK3CA mutations have never been described in ameloblastoma, but each of the observed mutations has been identified in several neoplasms and has been shown to have transforming activities (38-43).

A SMARCB1 R377H mutation was identified in three cases of ameloblastomas, two that also carried a BRAF V600E mutation and one 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 MCF7. Interestingly, vemurafenib appears to have a more potent effect in AM-1 cells than in the melanoma cell line with a homozygous BRAF V600E mutation as the IC₅₀ for AM-1 was 119nM compared to 476nM for SK-MEL-28. These findings suggest
a potential role for BRAF inhibitors in ameloblastoma treatment. While ameloblastoma is typically treated surgically, surgical resection often results in significant facial deformity and recurrences are common. In addition, pharmacological 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 while 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.

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FIGURE LEGENDS

**Figure 1.** A, Flow chart illustrating evaluation of ameloblastoma cases. B, Histopathology of the index case in which the *BRAF* V600E mutation was initially discovered. Initial mandible resection (top) and subsequent lymph node metastases (bottom). Metastases also occurred within the right lower lobe of lung and the second thoracic vertebrae (not shown). C, Sequence electropherograms demonstrating the presence of *BRAF* V600E mutation in the tumor of the index case (top) and absence in germline DNA (bottom). The asterisk indicates the c.1799 T>A, p.V600E mutation. D, Immunohistochemical staining using the VE1 antibody in a case of ameloblastoma with a *BRAF* V600E mutation (left) and in a case with wild-type *BRAF* (right) as demonstrated by allele-specific PCR, Sanger sequencing and next-generation sequencing.

**Figure 2.** Prevalence of mutations in ameloblastomas. A, Prevalence of *RAS-BRAF* and other mutations in 50 ameloblastoma cases. B, Distribution of *BRAF*, *KRAS*, *HRAS*, *NRAS*, *SMO*, *PIK3CA*, *CTNNB1* and *SMARCB1* mutations. Colored boxes indicate the presence of mutations in the indicated genes (rows) and samples (columns).

**Figure 3.** Clinical and prognostic significance of the *BRAF* V600E mutation and other variables in ameloblastoma. A, Box-plot showing a statistically significant difference in age distribution for *BRAF* V600E mutated and *BRAF* wild-type ameloblastomas (p=0.0007). Diamond indicated the mean, middle horizontal line indicates the median, box indicates 25th and 75th percentiles, and whiskers indicate minimum and maximum. B, Recurrence-free survival (in years) for *BRAF* V600E mutated and *BRAF* wild-type ameloblastomas using Kaplan-Meier method. C, Recurrence-free survival (in years) for mandibular and maxillary tumors using Kaplan-Meier method. D, Recurrence-free survival (in years) for tumors removed by resection and enucleation using Kaplan-Meier method. In (B), (C) and (D), p values were calculated using the Log-rank test.

**Figure 4.** *In vitro* effect of B-Raf inhibition. A, Electropherograms showing the *BRAF* c.1799T>A, p.V600E mutation (asterisk) in the ameloblastoma cell line AM-1 and the melanoma cell line SK-MEL-28. No *BRAF* mutations are identified in MCF7 cells. B, Effect on the phosphorylation of ERK and MEK in AM-1, SK-MEL-28 and MCF7 cells in response to vehicle alone or vemurafenib at 150nM, 500nM or 1000nM after 2 hours and 6 hours. C, Relative proliferation of AM-1, SK-MEL-28 and MCF7 cells in 1000nM vemurafenib. D, Dose-response curves for AM-1 at 216 hours (left; IC$_{50}$=119nM) and SK-MEL-28 at 96 hours (right; IC$_{50}$=476nM).
Table 1. Clinical characteristics of patient with *BRAF* V600E mutated and wild-type ameloblastoma

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

Abbreviations: cm, centimeters; 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>
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</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>0.989 (0.954, 1.024)</td>
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<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>

Abbreviations: CI, confidence interval
Patients with ameloblastoma from last fifteen years (84 patients)

Specimens not subjected to decalcification (50 patients)
- Evaluation using Ion AmpliSeq Cancer Hotspot panel, BRAF V600E allele-specific PCR and VE1 immunohistochemistry
- Mutations confirmed by Sanger sequencing of tumor and matched normal DNA

Specimens subjected to decalcification (34 patients)
- Evaluation using VE1 immunohistochemistry

Figure 1
Figure 3
Figure 4

(A) Sequence analysis of AM-1, SK-MEL-28, and MCF7 cell lines.

(B) Western blot analysis of ERK, MEK, p-ERK, and p-MEK in AM-1, SK-MEL-28, and MCF7 cells treated with different concentrations of AM-1 over 2 and 6 hours.

(C) Graph showing relative growth over time for AM-1, SK-MEL-28, and MCF7 cell lines.

(D) Graph showing the concentration-response relationship for AM-1 and SK-MEL-28 cell lines.

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