

High frequency of BRAF V600E mutations in ameloblastoma

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Abstract

Ameloblastoma is a benign but locally infiltrative odontogenic neoplasm. Although ameloblastomas rarely metastasise, recurrences together with radical surgery often result in facial deformity and significant morbidity. Development of non-invasive therapies has been precluded by a lack of understanding of the molecular background of ameloblastoma pathogenesis. When addressing the role of ERBB receptors as potential new targets for ameloblastoma, we discovered significant EGFR over-expression in clinical samples using real-time RT-PCR, but observed variable sensitivity of novel primary ameloblastoma cells to EGFR-targeted drugs *in vitro*. In the quest for mutations downstream of EGFR that could explain this apparent discrepancy, Sanger sequencing revealed an oncogenic BRAF V600E mutation in the cell line resistant to EGFR inhibition. Further analysis of the clinical samples by Sanger sequencing and BRAF V600E-specific immunohistochemistry demonstrated a high frequency of BRAF V600E mutations (15 of 24 samples, 63%). These data provide novel insight into the poorly understood molecular pathogenesis of ameloblastoma and offer a rationale to test drugs targeting EGFR or mutant BRAF as novel therapies for ameloblastoma.

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Introduction

Ameloblastoma is a slow-growing but locally infiltrative odontogenic neoplasm of the jaws [1,2], whose aetiology and pathogenesis are not understood. Due to their often complex growth pattern, most ameloblastomas are treated by surgical resection, often resulting in facial deformity and significant morbidity. More conservative approaches tend to result in recurrence and further surgery. Targeted therapy could potentially eliminate the need for extensive and/or repeated surgery.

ERBB receptors are a receptor tyrosine kinase subfamily that includes the epidermal growth factor

receptor (EGFR), ERBB2, ERBB3 and ERBB4. ERBB receptors are indispensable for development and frequently dysregulated in human malignancies [3]. EGFR and its ligands, EGF and transforming growth factor- α (TGFA) are expressed in the odontogenic epithelium of normal developing teeth [4], and strong EGFR expression has also been detected in ameloblastoma [4–6].

Here, we analysed the expression of all ERBB receptors in clinical ameloblastoma samples, using real-time RT-PCR. We also studied the role of ERBB signalling and assessed the feasibility of ERBB-targeted therapeutics in novel primary ameloblastoma cell lines. Furthermore, we report a high frequency of oncogenic BRAF V600E mutations in clinical ameloblastoma

Table 1. Clinical information and BRAF mutation status of the ameloblastoma patients; cases arranged as in Figure 1

Sample no.	Age	Sex	Ethnicity	Location	Primary/recurrent	Histology	Notes	BRAF V600E	
								BRAF status	IHC
1	66	M	Caucasian	Right mandible	Primary	S/MC follicular	Enucleated	V600E	Pos
2	70	F	Asian	Left mandible	Primary	S/MC follicular	Enucleated	V600E	Pos
3	61	M	Caucasian	Right posterior mandible	Primary	S/MC plexiform	Resected	WT	Neg
4	27	F	Black-Somali	Middle mandible	Primary	S/MC follicular	Enucleated	V600E	Pos
5	24	F	Black	Right mandible	Primary	S/MC plexiform	Enucleated	V600E	Pos
6	50	F	Black	Left mandible	Recurrence	Plexiform	Resected, tumour sample decalcified in formic acid	V600E	Neg*
7	36	M	Afro-Caribbean	Left mandible	Residual	S/MC follicular	Enucleated	WT	Neg
8	47	M	Caucasian	Mandible	Primary	S/MC follicular	Resected	V600E	Pos
9	32	M	North African	Right posterior mandible	1st recurrence	S/MC plexiform	Primary tumour at age 27; enucleated	WT	NA
10	46	M	African	Middle anterior mandible	3rd recurrence	S/MC follicular	Primary tumour at age 25; radical resection	V600E	NA
11	14	M	Black-North African	Left posterior mandible	Primary	S/MC plexiform	Left angle and ramus	V600E	Pos
12	34	F	Caucasian	Left posterior mandible	1st recurrence	S/MC follicular	Resected	V600E	Pos
13	84	M	Caucasian	Right mandible	Primary	S/MC follicular	Resected	WT	Neg
14	18	F	Caucasian	Left anterior mandible	Primary	S/MC follicular	Enucleated	V600E	NA
15	16	M	Caucasian	Mandible	Primary	S/MC plexiform	Enucleated	V600E	Pos
16	61	F	NA	Right mandible	Primary	S/MC plexiform/follicular	Enucleated	WT	Neg
17	69	M	Caucasian	Left posterior mandible	Primary	S/MC plexiform	Primary tumour for sample 18; enucleated	WT	Neg
18	77	M	Caucasian	Left posterior mandible	1st recurrence	S/MC plexiform	Recurred after 8 years, primary tumour sample 17	WT	Neg
19	69	M	Caucasian	Right posterior mandible	Primary	S/MC plexiform	Patient died after operation	WT	Neg
20	43	M	Caucasian	Right body mandible	Primary	S/MC follicular	Enucleated	V600E	Pos
21	44	F	Black-African	Right posterior mandible	Primary	S/MC plexiform	No recurrence after 5 years	V600E	Pos
22	33	M	African	Middle anterior mandible	3rd recurrence	S/MC follicular	Primary tumour at age 20; enucleated	V600E	Pos
23	46	F	Afro-Caribbean	Left body mandible	1st recurrence	S/MC plexiform	Primary tumour at age 34; resected	V600E	NA
24	31	M	Black-African	Left posterior mandible	Primary	S/MC follicular	Recurred after 5 years, no real-time RT-PCR data available	WT	Neg

*Decalcification of ameloblastoma tumour samples using formic acid may hinder the BRAF V600E immunohistochemistry. S/MC, solid/multicystic; WT, wild-type; NA, data/sample not available; IHC, immunohistochemistry.

samples and demonstrate that BRAF V600E mutation was associated with resistance to EGFR-targeted drugs in primary ameloblastoma cells.

Materials and methods

Patients and tissue specimens

Fresh frozen tumour samples from 24 conventional intra-osseous ameloblastomas (Table 1), eight sporadic keratocystic odontogenic tumours (KCOT) and six samples of normal oral mucosa (see supplementary material, Table S1) were included in the study. Two ameloblastoma samples were from the primary and recurrent tumours of the same patient (samples 17 and

18; Table 1). Ethics Committee approvals (1–11 March 2007, 0/H0703/054 and CPP53-10) and the patients' written informed consents were obtained in accordance with the Helsinki Declaration.

Real-time RT-PCR

RNA isolation and real-time RT-PCR (TaqMan, Applied Biosystems) analyses were carried out as previously described [7,8]. The amount of tumour tissue in the samples was >90%. Primer and probe sequences have been described previously [8] or are described in Table S2 (see supplementary material). One ameloblastoma sample (patient sample 24) was not included in the analysis, due to insufficient RNA concentration.

Establishment of primary ameloblastoma cells and ameloblastoma fibroblasts

Fresh samples from ameloblastoma tumours (AB10, patient 3; ABSV, patient 12; see Table 1) were cut into small pieces (approximately 1 × 1 mm) and placed in T25 cell culture flasks with 1 ml CnT-24 medium (CELLnTEC), supplemented with Pen/Strep/Amphotericin B Solution (CELLnTEC). Outgrowing ameloblastoma cells were harvested at confluence and maintained in the CnT-24 medium. Primary ameloblastoma fibroblast (AF) cultures were established from an ameloblastoma sample (tumour not analysed in this study) as above, using Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal calf serum (FCS), 50 U/ml penicillin and 50 µg/ml streptomycin as the culture medium.

Antibodies and inhibitors

The EGFR antibodies cetuximab and panitumumab, as well as the ERBB2 antibody trastuzumab, were purchased from Turku University Hospital pharmacy. The EGFR tyrosine kinase inhibitors gefitinib and erlotinib were purchased from Santa Cruz Biotechnology. EGFR/ERBB4 inhibitor AG1478, PI3K inhibitor LY294002 and MEK inhibitor PD98059 were purchased from Calbiochem (Merck Millipore).

MTT cell viability assay

AB10 and ABSV cells were plated onto 96-well plates in triplicate, at a density of 3500 cells/well, in CnT-24 Oral Epithelium Medium, with or without inhibitors. After 72 h, the number of viable cells was estimated using the MTT assay (CellTiter 96 non-radioactive cell proliferation assay; Promega) according to the manufacturer's guidelines.

Western blotting

Cells growing on six-well plates were treated overnight with EGFR, ERBB2, AKT or MEK inhibitors or for 10 min with EGF (R&D). Samples of cell lysates (25 µg total protein) were analysed by western blotting with antibodies against phospho-EGFR, phospho-ERK, phospho-AKT and ERK (cat. nos 2220, 9101, 9271 and 9102, Cell Signaling Technology) and EGFR and AKT (sc-03 and sc-1618, Santa Cruz Biotechnology).

Targeted cDNA sequencing

Sequences coding for *EGFR* kinase domain and *KRAS*, *NRAS*, *HRAS* or *BRAF* genes were PCR-amplified and purified using NucleoSpin Gel and PCR Cleanup kit (Macherey-Nagel). Both strands of amplified fragments were Sanger-sequenced for recurrent mutations (kinase domain for *EGFR*, codons 12, 13 and 61 for *RAS* genes, codon 600 for *BRAF*). Primers used in cDNA sequencing are described in Table S2 (see supplementary material).

BRAF V600E immunohistochemistry

Immunohistochemical staining was performed using BRAF V600E mutation-specific antibody VE1 (Spring Biosciences), as previously described [9].

Statistical analyses

Real-time RT-PCR data were analysed by one-way ANOVA, using the Games–Howell *post hoc* test. MTT cell viability assays were analysed by *t*-test. To analyse the relationships between *BRAF* mutation status and clinical patient data, Fisher's exact test was used. Association of *BRAF* mutation status with *EGFR* expression (high or low; above or below median expression, respectively) was analysed using Fisher's exact test. Statistical analyses were carried out using SPSS statistics v 20 (IBM).

Results

EGFR and *ERBB4* are over-expressed in ameloblastoma

A real-time RT-PCR analysis of 23 solid/multicystic ameloblastomas (patient samples 1–23; Table 1) was performed to study the expression of *ERBB* receptors. Eight KCOTs and six normal oral mucosa samples were included in the analysis as controls (see supplementary material, Table S1). *EGFR* and *ERBB4* were specifically over-expressed in ameloblastoma when compared to normal samples (*EGFR*, $p = 0.003$; *ERBB4*, $p = 0.01$) or to KCOT (*EGFR*, $p = 0.001$; *ERBB4*, $p < 0.001$) (Figure 1A, D). *EGFR* over-expression is in accordance with previous studies reporting high EGFR protein levels in ameloblastoma [4–6]. The predominantly expressed ERBB4 receptor isoforms in ameloblastoma were the JM-a isoforms (see supplementary material, Figure S1). For *ERBB2*, no statistically significant differences were observed (Figure 1B). *ERBB3* was significantly more highly expressed in KCOT than in ameloblastoma ($p = 0.011$) (Figure 1C).

Establishment of ameloblastoma cell lines

To address the function of ERBB receptors in ameloblastoma, two non-immortalized primary ameloblastoma cell lines, AB10 and ABSV, were established from patient samples 3 and 12, respectively (Table 1). A primary fibroblast cell line (ameloblastoma fibroblasts, AFs) was also established (from a tumour not analysed in this study). AB10 and ABSV cells were morphologically identical and formed an epithelial-like monolayer very similar to those of two previously published ameloblastoma cell lines [10,11], whereas ameloblastoma fibroblasts demonstrated a typical spindle-shaped fibroblastic morphology (Figure 2A). The ameloblastoma cells expressed high levels of epithelial markers *KRT14* (keratin 14), *KRT19* (keratin 19) and *CDH1* (E-cadherin)

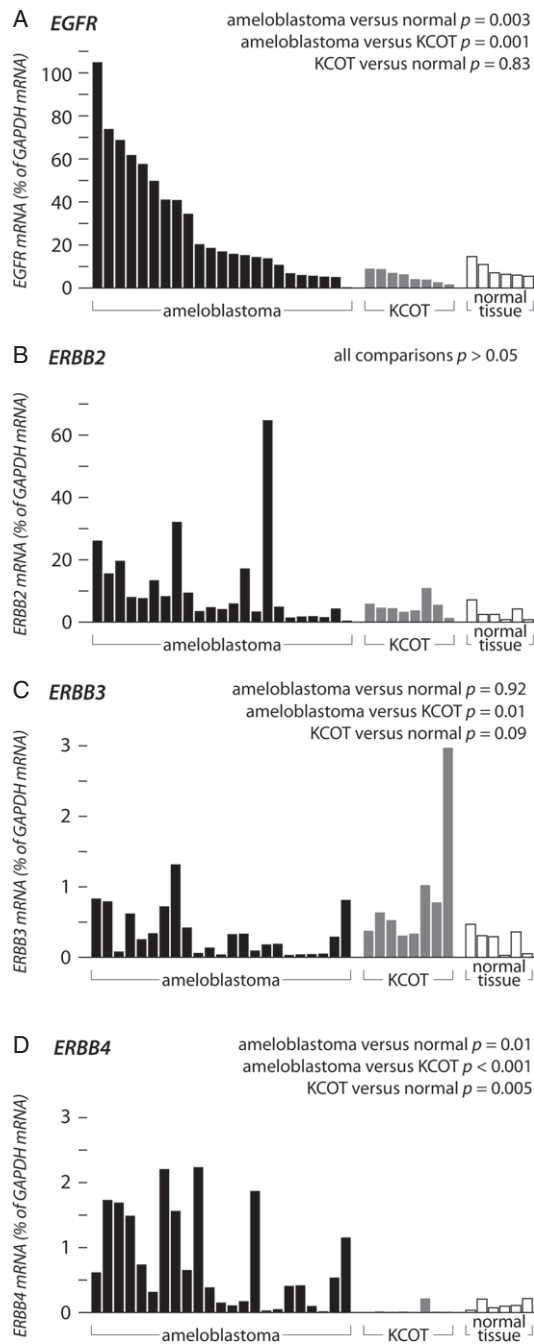


Figure 1. Real-time RT-PCR analysis of *ERBB* receptor expression in ameloblastoma, keratocystic odontogenic tumour (KCOT) and normal oral mucosa. Twenty-three ameloblastomas, eight KCOTs and six normal samples were analysed for *EGFR* (A), *ERBB2* (B), *ERBB3* (C) or *ERBB4* (D) expression.

(Figure 2B), whereas the expression of mesenchymal markers *CDH2* (N-cadherin) and *VIM* (vimentin) was almost undetectable (Figure 2B). The *ERBB* receptor expression pattern was similar in both ameloblastoma cell lines (Figure 2D) and corresponded to that observed in the ameloblastoma tumour samples (Figure 1). However, neither of the cell lines expressed detectable levels of *ERBB4*, although *ERBB4* was expressed in the original tumour from which the AB10 cell line was established. This suggests that *ERBB4* expression was lost during cell line establishment.

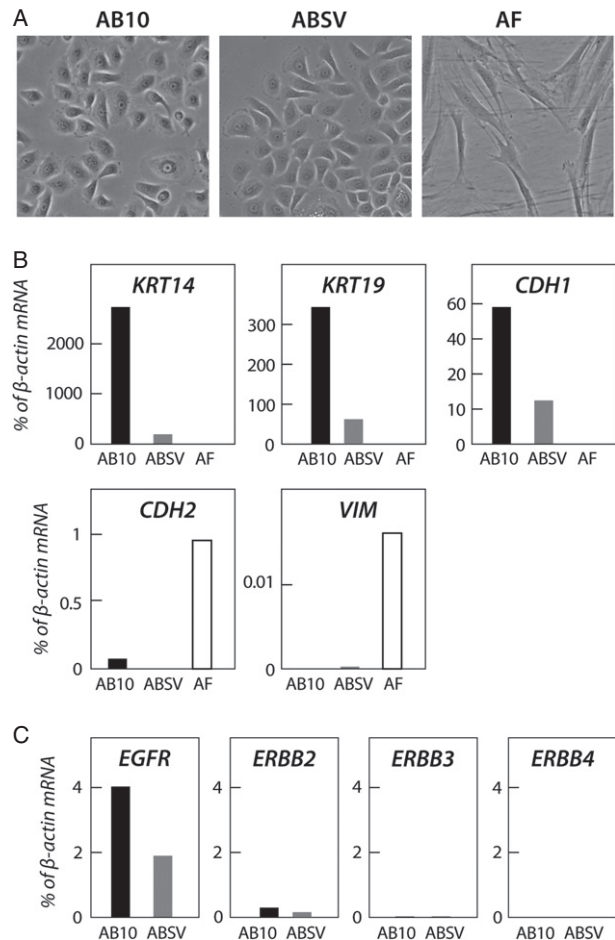


Figure 2. Characterization of established primary ameloblastoma tumour cell lines. (A) Established AB10, ABSV and ameloblastoma fibroblast cultures were grown on six-well plates and photographed using $\times 200$ magnification. (B) The cell lines were analysed for the expression of epithelial markers *KRT14* (keratin 14), *KRT19* (keratin 19) and *CDH1* (E-cadherin) and mesenchymal markers *CDH2* (N-cadherin) and *VIM* (vimentin), using real-time RT-PCR. (C) AB10 and ABSV cell lines were analysed for *EGFR*, *ERBB2*, *ERBB3* and *ERBB4* expression, using real-time RT-PCR.

EGFR inhibition in ameloblastoma cells

The effect of EGFR inhibition on the proliferation of primary ameloblastoma cells was analysed by MTT cell viability assays. In AB10 cells, 72 h of treatment with the EGFR antibodies cetuximab and panitumumab already promoted a significant, dose-dependent reduction in proliferation at a concentration of 0.1 $\mu\text{g/ml}$ ($p < 0.001$) (Figure 3A). Consistently, the EGFR tyrosine kinase inhibitors (TKI) erlotinib, gefitinib and AG1478 already significantly suppressed AB10 cell growth at the concentration of 0.01 μM ($p < 0.001$, $p = 0.004$, and $p < 0.001$, respectively). However, similar treatment with the ERBB2-antibody trastuzumab did not demonstrate a dose-dependent effect (Figure 3A). Treating AB10 cells with EGFR-targeted antibodies or TKIs abolished EGFR phosphorylation and silenced the RAS-RAF-MAPK and PI3K-AKT signalling pathways downstream of EGFR, as seen by a reduction of ERK and AKT phosphorylation after treatment (Figure 3B). Consistent with the proliferation assays,

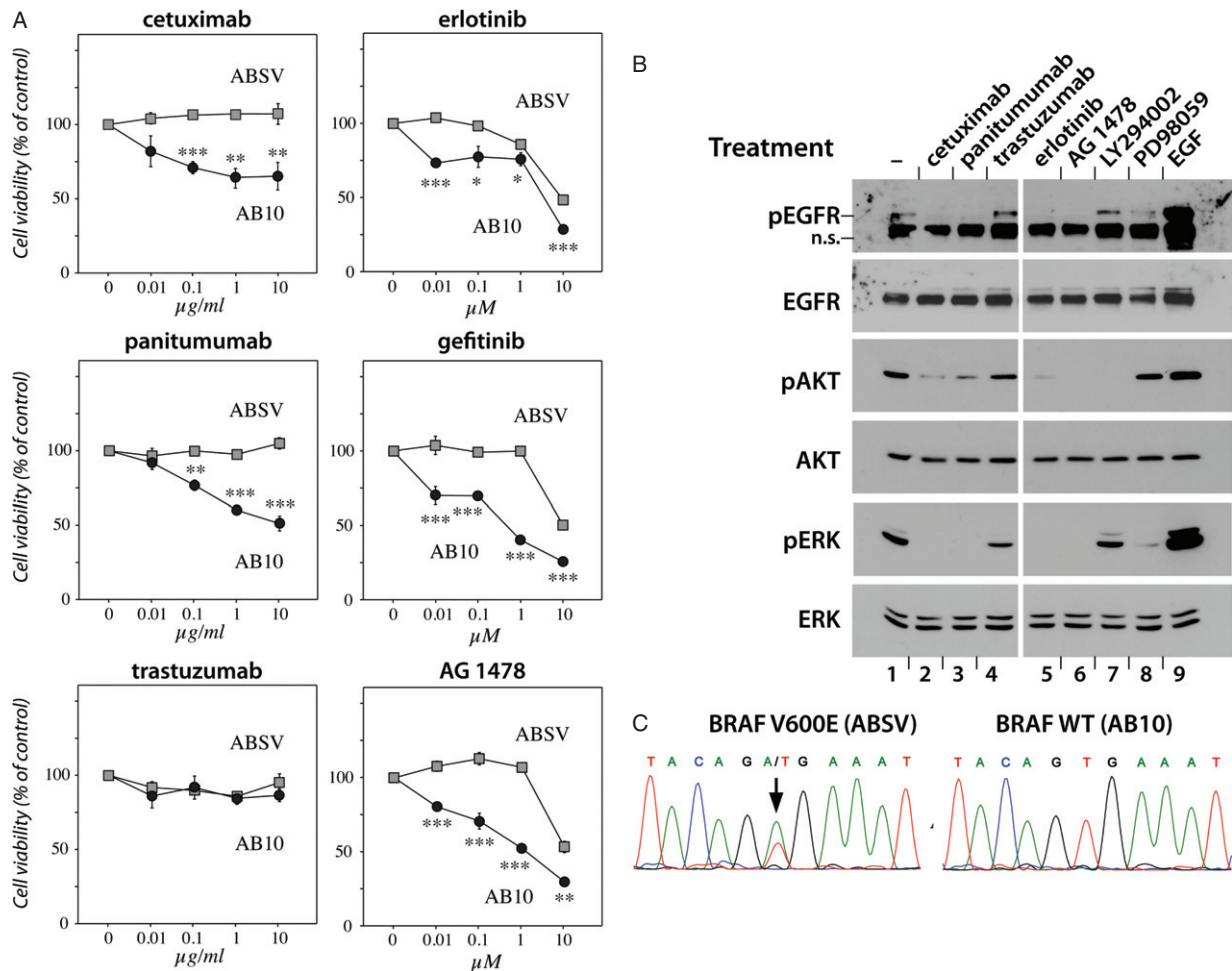


Figure 3. Effect of EGFR inhibition on ameloblastoma cells. (A) AB10 and ABSV cells were plated on 96-well plates and incubated for 72 h, with or without the indicated concentrations of EGFR monoclonal antibodies cetuximab or panitumumab, ERBB2 monoclonal antibody trastuzumab or EGFR tyrosine kinase inhibitors erlotinib, gefitinib or AG1478. Cell viability after incubation was estimated using the MTT assay. *Significant differences between the proliferation of AB10 and ABSV cells (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; Student's t -test). (B) AB10 cells were grown on six-well plates and treated overnight with cetuximab (10 $\mu\text{g/ml}$), panitumumab (10 $\mu\text{g/ml}$), trastuzumab (10 $\mu\text{g/ml}$), erlotinib (10 μM), AG1478 (10 μM), PI3-K inhibitor LY294002 (10 μM) or MEK inhibitor PD98059 (10 μM) or for 10 min with EGF (50 $\text{ng}/\mu\text{l}$). After treatment, phosphorylation of EGFR, AKT and ERK was analysed by western blotting, using phospho-specific antibodies (n.s., non-specific band). Total EGFR, AKT and ERK levels were determined using anti-EGFR, anti-AKT and anti-ERK antibodies. (C) The coding region of the *BRAF* gene was PCR-amplified from AB10 and ABSV cell cDNA, followed by Sanger sequencing of both strands of a 320 bp amplicon flanking the V600 codon of *BRAF*. The arrow indicates the mutated nucleotide responsible for the V600E substitution.

ERBB2 inhibition had no major effect on the phosphorylation of ERK or AKT. When tested on the ABSV cells, however, neither panitumumab nor cetuximab had a significant effect on proliferation (Figure 3A). The EGFR TKIs reduced proliferation of ABSV cells by 50%, but the effect was achieved only with the highest concentration of 10 μM . Indeed, there was a clear, statistically significant difference in the sensitivity of AB10 and ABSV cell lines to EGFR inhibition (Figure 3A).

BRAF mutation is associated with resistance to EGFR-targeted drugs in primary ameloblastoma cells

Specific genetic markers predict the response to EGFR-targeted cancer drugs in the clinic and *in vitro* [12–20]. To test whether the differences observed in

the responses to EGFR-targeted drugs in ameloblastoma cells were due to genetic factors, cDNA sequencing was performed to reveal mutations in the *EGFR* kinase domain or in the RAS–RAF–MAPK signalling pathway genes *KRAS*, *NRAS*, *HRAS* and *BRAF*. No mutations were observed in the *EGFR* kinase domain or in the *RAS* genes in either of the two cell lines. However, the unresponsive ABSV cell line harboured a *BRAF* V600E mutation (Figure 3C), caused by a point mutation 1799T > A. These findings demonstrate that the resistance of the ABSV cell line to EGFR-targeted drugs was associated with an activating mutation in a downstream signalling pathway.

BRAF mutations are frequent in ameloblastoma

Twenty-four ameloblastoma samples were analysed for *BRAF* mutations by cDNA sequencing. *BRAF*

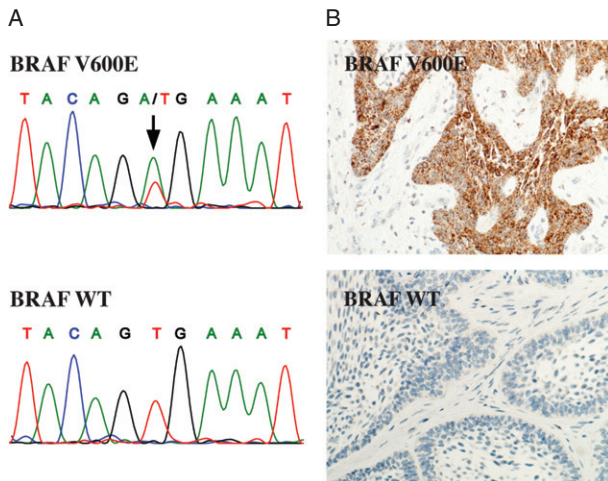


Figure 4. Analysis of BRAF V600E mutations in ameloblastoma tumour samples. (A) The coding region of the *BRAF* gene was PCR-amplified from tumour cDNA, followed by Sanger sequencing of both strands of a 320 bp region flanking the V600 codon of *BRAF*; arrow indicates the mutated nucleotide responsible for the V600E substitution. (B) Immunohistochemistry was performed for tumour sections using BRAF V600E-specific monoclonal antibody. Matching electropherograms and images from BRAF V600E immunohistochemistry are shown for BRAF V600E-positive (top, patient sample 20) and negative (bottom, patient sample 24) tumours.

V600E-specific immunohistochemistry was also performed for cases where appropriate tumour sections were available. Of 24 tumours, 15 (63%) were observed to harbour the BRAF V600E (1799T > A) mutation (Figure 4, Table 1). In most cases, as in the ABSV cell line (Figure 3C), the mutation appeared heterozygous (Figure 4A). However, as the sequencing was performed from cDNA and the involvement of normal tissue could not be totally excluded, gene-level zygosity of the mutation could not be comprehensively addressed in the tumour samples. The mutations were found in patients with different ethnicities, including individuals of Caucasian ($n = 6$), Black-African ($n = 7$), Afro-Caribbean ($n = 1$) and Asian ($n = 1$) origin, with no significant association to specific ethnicity. *BRAF* mutation status also did not correlate with the sex or age of the patients, tumour histology, tumour recurrence or *EGFR* expression level ($p > 0.1$). As expected, the original tumour from which the AB10 cell line was established (patient sample 3; Table 1) was negative for the mutation and the tumour from which the ABSV line was established (patient sample 12; Table 1) was positive. These findings suggest the presence of a high-frequency oncogenic mutation in clinical ameloblastoma.

Discussion

The pathogenesis of ameloblastoma has remained elusive, despite considerable efforts to characterize the underlying molecular mechanisms. Here, the ERBB

signalling in ameloblastoma was studied using clinical ameloblastoma samples as well as novel ameloblastoma cell lines. Real-time RT-PCR analysis of *ERBB* expression demonstrated a statistically significant overexpression of *EGFR* and *ERBB4* in ameloblastomas ($n = 23$) when compared to clinical samples of KCOT ($n = 8$) or to normal mucosa ($n = 6$). Pharmacological inhibition of EGFR suppressed the proliferation of one primary ameloblastoma cell line (AB10) but was ineffective for the other (ABSV). In the quest for factors underlying this difference, a BRAF V600E mutation was found in the resistant ABSV cell line. These observations demonstrate that, similar to *KRAS* mutations in colorectal cancer [12,17,18,20], the BRAF V600E mutation is associated with resistance to EGFR-targeted drugs in ameloblastoma cells. BRAF V600E mutations were found in 63% (15 of 24) of the ameloblastoma samples. The high incidence of *BRAF* mutations represents the first indication of a high-frequency oncogenic mutation in ameloblastoma. Given the high response rate of BRAF V600E-positive metastatic melanoma to mutant BRAF-targeted inhibition by vemurafenib [21] or dabrafenib [22], our observations also offer a rationale for future testing of BRAF-targeted therapeutics for ameloblastoma.

The presence of activating *BRAF* mutations suggests the involvement of a hyperactive RAS-RAF-MAPK pathway in the pathogenesis of ameloblastomas. The observed dependency of AB10 cells on EGFR signalling supports this hypothesis, as the RAS-RAF-MAPK pathway is well known to operate downstream of EGFR [23], and as ERK phosphorylation was completely abolished in response to EGFR inhibition in these cells. Interestingly, transgenic mice carrying the *vHa-RAS* oncogene, a constitutively active homologue of the human *HRAS*, develop odontogenic tumours that closely resemble human ameloblastoma [24,25].

Taken together, our results indicate that a hyperactive RAS-RAF-MAPK pathway is closely associated with ameloblastoma pathogenesis, either through EGFR-mediated signalling or through frequent activating mutations in the *BRAF* gene. Thus, our observations offer for the first time a rationale for designing targeted therapies in ameloblastoma.

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Author contributions

KK, KE and KH designed the study; KH, PRM, BR and JK provided the clinical ameloblastoma samples; KH and PRM diagnosed the tumours; JC and PRM established the AB10 cell line; AR performed *BRAF* V600E immunohistochemistry; KK conducted all the remaining experimental work; and KK, KE and KH prepared the manuscript.

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SUPPLEMENTARY MATERIAL ON THE INTERNET

The following supplementary material may be found in the online version of this article:

Figure S1. Real-time RT–PCR analysis of the expression of *ERBB4* receptor isoforms in ameloblastoma, keratocystic odontogenic tumour (KCOT) and normal oral mucosa.

Table S1. Clinical information for KCOT patients and normal sample donors.

Table S2. Primer and probe sequences used in real-time RT–PCR and targeted cDNA sequencing.