Journal of
Dental Research

The Mutational Profile of Unicystic Ameloblastoma

Journal:	*Journal of Dental Research*
Manuscript ID	JDR-18-0349.R1
Manuscript Type:	Research Reports
Date Submitted by the Author:	n/a
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Keywords:	Biomarkers, Bioinformatics, Cancer biology
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The Mutational Profile of Unicystic Ameloblastoma

Short title: Oncogenic mutations in unicystic ameloblastoma

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Abstract 235 words, manuscript 3197 words, 6 keywords, 5 figures, 30 references, 4 supplementary tables, 3 supplementary figures

Abstract

BRAF V600E is the most common mutation in conventional ameloblastoma (AM) of the mandible. In contrast, maxillary AMs appear to harbor more frequently *RAS, FGFR2* or *SMO* mutations. Unicystic ameloblastoma (UAM) is considered a less aggressive variant of ameloblastoma, amenable to more conservative treatment, and classified as a distinct entity. The aim of this study was to characterize the mutation profile of UAM (N=39) and to compare it to conventional AM (N=39). The associations between mutation status and recurrence probability were also analyzed. In the mandible, 94% of UAMs (29/31 including 8/8 luminal, 6/8 intraluminal and 15/15 mural subtypes) and 74% of AMs (28/38) revealed *BRAF* V600E mutations. Among the *BRAF* wild-type cases, one UAM showed a missense SMO mutation (p.L412F) whereas two NRAS (p.Q61R), two HRAS (p.Q61R) and two FGFR2 (p.C383R) ~~single hotspot~~ activating mutations were identified in AM. Of the three maxillary UAMs, only one revealed a *BRAF* V600E mutation. Taken together, our findings demonstrate high frequency of activating *BRAF* V600E mutations in both UAM and AM of the mandible. In maxillary UAMs, the *BRAF* V600E mutation prevalence appears to be lower as was shown for AM previously. It could therefore be argued that UAM and AM are part of the spectrum of the same disease. AMs without *BRAF* V600E mutations were associated with an increased rate of local recurrence (p-value 0.0003) which might indicate that routine mutation testing also has an impact on prognosis.

Keywords: ameloblastoma, drug therapy, genetic markers, MAP kinase signaling; odontogenic tumors, BRAF

Introduction

Four years ago, we reported *BRAF* V600E mutations in 63% (15/24) of conventional ameloblastomas (AMs), introducing aberrant MAP-kinase signaling as underlying the majority of AMs (Kurppa et al. 2014; Heikinheimo et al. 2015). We also demonstrated that *BRAF* mutation-negative non-immortalized primary AM cells were sensitive to EGFR-targeted drugs. These observations suggested that two groups of AM patients exist: *BRAF* mutation-negative patients who might benefit from EGFR inhibition, and *BRAF* mutation-positive patients who could potentially benefit from BRAF-targeted therapies (Gomes et al. 2014; Heikinheimo et al. 2015). Brown and colleagues additionally found that *BRAF* wild-type tumors recur more rapidly (Brown et al. 2014).

In addition to the high incidence of activating *BRAF* mutations, mutually exclusive and less common mutations in other MAP-kinase genes such as *KRAS*, *NRAS* as well as *HRAS* and *FGFR2* have been reported in AM (Brown et al. 2014; Sweeney et al. 2014). These genes mediate cell proliferation, differentiation and survival and are commonly activated in various human malignancies (Holderfield et al. 2014). In addition to mutations in the MAP-kinase cascade, a high incidence of activating mutations in the *SMO* gene has been reported in AM, particularly in maxillary tumors (Brown et al. 2014; Sweeney et al. 2014). SMO is a transmembrane activator of the hedgehog signaling pathway, which is commonly mutated in basal cell carcinoma and occasionally in odontogenic keratocyst (Kim et al. 2013). Rarely detected mutations in AM include *PIK3CA*, *SMARCB1* and *CTNNB1* (Brown et al. 2014; Sweeney et al. 2014).

While AM represents a benign but locally aggressive odontogenic neoplasia that can recur following incomplete excision, the unicystic subtype (UAM) remains a matter of ongoing debate (Wright el al. 2014). The current WHO Classification of Head and Neck tumors (2017) concurs with the original description of UAM as representing a prognostically distinct entity comprising three distinct subtypes: luminal, intraluminal and mural (Robinson and Martinez 1977; Vered et al., 2017). The luminal and intraluminal UAM variants are generally regarded as the least aggressive form whereas the mural type seems to recur at similar rates to AM. Hence, some experts regard it as an early version of AM (Li et al. 2000; Wright et al. 2014).

Whilst the mutational profile of AM has been analyzed intensively, there is little data on the molecular background of UAM (Diniz et al. 2015; Pereira et al. 2016). We therefore

analyzed UAMs for driver mutations and compared the results to those obtained from a similar number of AM cases. To further elucidate the clinical significance of the mutation testing, we have analyzed results for treatment modality (enucleation or resection), follow-up time (months) and outcome (recurrence or no recurrence).

Materials and Methods

Patients and Tissue Specimens

Seventy-eight formalin-fixed and paraffin-embedded (FFPE) ameloblastoma samples, consisting of 39 unicystic ameloblastomas (UAM) and 39 conventional ameloblastomas (AM) were included in the study. These were selected after careful histopathological re-evaluation by pathologists KH, PRM, CK, GW, and DB (Appendix/Tables 1 and 2). The tissue blocks were collected from Oral Pathology and/or Pathology Departments in Finland, Denmark, Sweden, the United Kingdom, and Switzerland. Out of these 78 cases, fresh tumor specimens were additionally available from 24 AM cases as described previously (Kurppa et al. 2014). Clinical patient data (Appendix/Tables 1 and 2) and follow- up information were obtained from patients' medical records (Appendix/Tables 3 and 4). Approvals from the Ethics Committees (1/11 March 2007, 0/H0703/054 and CPP53-10) and patients' written informed consents in relation to the fresh tumor specimens were obtained in accordance with the Helsinki Declaration.

RNA and DNA Extraction

Cryosections of the fresh surgical specimens and sections from the FFPE tissue blocks were reviewed prior to RNA and DNA extraction to confirm that over 90% of the tissue represented tumor. Total RNA was isolated from fresh AM (N=24) tissue samples as described earlier (Heikinheimo et al. 1991). DNA was isolated from FFPE UAM (N=39) and AM (N=15 tissue samples after using the QIAamp DNA Mini Kit (Qiagen, Venlo, the Netherlands) according to the manufacturer's instructions as previously reported (Thiel et al. 2013). Flow chart of the study is given in Appendix/Figure 1.

BRAF V600E Genotyping

Genotyping was performed as described previously (Thiel et al. 2013). Quantitative RT-PCR (qPCR) genotyping was used to detect *BRAF* V600E mutation (NM_004333.4(BRAF): c.1799T>A, pVal600Glu in UAM (N=39) and AM (N=14) samples. Primers and probes to

detect *BRAF* wild-type and V600E have been described previously (Benlloch et al. 2006). Each sample was processed in duplicate with the 7500 Fast Real-Time PCR machine (Thermo Fisher Scientific, Waltham, MA, USA). The total reaction volume was 10 µl per well and the genotyping cycling conditions were: 60°C for 1 minute, 95°C for 10 minutes, followed by 40 cycles of 95°C denaturation for 15 seconds, and 60 °C annealing for 1.5 minutes. After 40 cycles, post-PCR reading occurred at 60°C for 1 minute. Genomic DNA from HT-29 cells was used as a positive control. The delta Ct (Ct mutation – Ct wild-type; threshold 0.05) limit was 6.5, and Ct values above 36 were disregarded.

Sanger Sequencing

Twenty-four mandibular AMs were analyzed for recurrent mutations in KRAS, NRAS and HRAS genes (codons 12, 13 and 61) by cDNA sequencing. cDNA synthesis and Sanger sequencing of KRAS, NRAS and HRAS were performed as previously described (Kurppa et al. 2014). Sanger sequencing was also used to validate mutations identified by semiconductor sequencing.

Next-Generation Sequencing

Ion torrent semiconductor sequencing was used for the analysis of four UAMs (12,13,17 and 29) and six AM cases (3, 13, 16, 26, 35 and 39), respectively, in which BRAF, NRAS or HRAS mutations were not identified (Appendix/Tables 1 and 2). The Ion AmpliSeq™ Comprehensive Cancer Panel was used in conjunction with the AmpliSeq Library Kit 2.0 to capture exons of 143 genes from the Cancer Gene Census database and the resulting libraries were run on the Ion PGM™ Sequencer. The raw reads were processed using Ion Reporter software with standard settings.

Variant Detection and Filtering

Sequence reads were mapped onto the human genome hs37d5 using the BWA software. We then used the GATK haplotype caller with standard settings, followed by Variant Quality Score Recalibration for single-base substitution identification and the Scalpel algorithm for indels. The 2017 versions of ANNOVAR databases were used for variant annotation. Clinical significance of variant calls was assessed using the consensus mutation classification criteria of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology (Richards 2015).

Immunohistochemistry

Mouse monoclonal antibodies to human BRAF p.V600E (clone VE1; 1:2000) and rabbit monoclonal antibodies to human RAS p.Q61R (clone SP174; 1:50), both from Spring Bioscience, Pleasanton, CA, USA were used. Three-micron sections were cut from undecalcified FFPE blocks, processed and stained with Ventana BenchMark XT immunostainer (Ventana Medical Systems, Tucson, Az, USA). BRAF mutations were visualized using OptiView DAB IHCv3 (Ventana) and RAS mutations with UltraViewRed (Ventana). The sections were counterstained with hematoxylin.

Statistical Analysis

Kaplan–Meier curves (Therneau and Grambsch, 2000; Rich et al. 2010) were calculated to analyze the recurrence-free survival probability in patients after treatment based on follow-up information. The Kaplan-Meier analysis was conducted using the statistical software R survival package (Therneau, 2015). To establish statistical significance the Log-Rank Test p-value was computed using the *survdiff* function in the R package "survival".

Kaplan–Meier analyses were carried out on 33 UAMs and 19 AMs, for which the following data was available: treatment (enucleation vs resection), follow-up time (months), and outcome (recurrence or no recurrence). For patients who underwent multiple treatments (8 UAMs and 7 AMs), each observation was treated as a separate data point in the analysis.

Multiple Kaplan–Meier analyses were carried out comparing two selected non-overlapping groups, UAMs and AMs, in each analysis. The two groups were compared in relation to *BRAF* V600V vs. wild type, mandible vs. maxilla, and resection vs. enucleation. Comparisons were made between the AM and UAM groups against each other as a whole, the mandibular AM *BRAF* V600E positive group against the mandibular UAM *BRAF* V600E positive group and the mandibular AM *BRAF* V600E positive group against the mandibular mural *BRAF* V600E positive UAMs.

Odds ratio (Martin and Altman 2000) was calculated using the Wald method to compare the likelihood of recurrence between the *BRAF* V600E and wild-type ameloblastomas using functionality provided by the statistical software R fmsb package (https://cran.r-project.org/web/packages/fmsb/) In addition to the empirical odds ratio, the 95% confidence interval and p-value were computed.

Results

Clinical data

The UAM and AM clinico-pathological data and mutation status are summarized in Figure 1 and Appendix/Tables 1 and 2 and Appendix/Figure 2.

In the UAM series, 21/39 were female and 18/39 were male, and in the AM series 18/39 were female and 21/39 male. 90% (35/39) of the UAMs and 97% (38/39) of the AMs were located in the mandible. The mean age at the time of diagnosis was 29.9 years for UAM patients (range 7-85 years) and 39.4 years for AM patients (11-84 years).

Follow-up Information

Follow-up time and outcome (recurrence or no recurrence) were retrieved from the medical records of 33/39 UAM and 21/39 AM patients (Appendix/Tables 3 and 4 and Appendix/Figure 2). The follow-up time was 8.2 years on average for the UAMs and 11.7 years for the AMs. 14/33 UAMs (12 in the mandible and two in the maxilla) and 17/21 AMs (16 in the mandible and one in the maxilla) were reported to have one or multiple recurrences.

The time-to-first recurrence information (average 6.1 years) was available for 12/33 UAM patients. Five represented intraluminal, two luminal, and five mural subtypes. Eleven of the primary UAMs were treated by enucleation and one by resection.

The time-to-first recurrence information (average 7.8 years) was available for 14/21 AM patients. Eleven represented follicular, nine plexiform, and one acanthomatous subtypes. 7/14 of the primary AMs were treated by enucleation and 7/14 by resection. The first recurrence following enucleation occurred after an average of 6.2 years, that following resection after 8.6 years.

Mutations in UAM and AM

The quality of DNA was found to be suitable for sequencing in 34/39 UAM and in 39/39 AM samples. The cancer-driving mutations detected are listed in Figure 1, Appendix/Tables 1 and 2 and Appendix/Figure 2.

In the mandible, 94% (29/31) of the UAMs were *BRAF* V600E positive: 8/8 luminal, 6/8 intraluminal and 15/15 mural subtypes. In two mandibular intraluminal *BRAF* mutation-

negative UAMs, one harbored a *SMO* (p. L412F) mutation, but in the other case no mutation was identified. In the maxilla 33% (1/3) were *BRAF* V600E positive and 67% (2/3) were *BRAF* V600E wild types.

Seventy-four percent of the mandibular AMs were *BRAF* V600E positive (28/38). In addition, two NRAS c.182A>G (5%) and two HRAS c.182A>G (5%) single-nucleotide substitutions were detected (Figures 1 and 2). In both genes, the mutations corresponded to an amino acid substitution Q61R, which is a well-known activating mutation (Buhrman et al. 2010). Two *FGFR2* (p.C383R; 5%) mutations were detected in AM by targeted, next-generation sequencing and were further confirmed by Sanger sequencing (Figures 1 and 2). HRAS, BRAF and FGFR2 mutations were mutually exclusive. Four mandibular AMs were *BRAF*, *RAS* and *FGFR2* negative. In the maxilla, one AM was *BRAF* mutation-negative.

Associations of *BRAF* V600E mutations with age

The *BRAF* V600E mutation status has been suggested to be associated with younger age in the ameloblastoma patients (Brown et al. 2014). The small number of *BRAF* V600E wild-type (N=4) UAMs precluded statistical analysis. For the *BRAF* V600E positive AMs, the mean age at the time of diagnosis of the primary tumor was 36.2 years. Patients with wild-type tumors had a mean age of 47.2 years at diagnosis. We were not able to establish statistical significance between the mean ages.

~~Eighty-three percent (10/12) of the recurred UAMs carried a *BRAF* V600E mutation and 8% (1/12) were *BRAF* negative. 57% (8/14) of the recurred AMs were *BRAF* V600E positive and 43% (6/14) *BRAF* V600E were wild type.~~

Immunohistochemistry

BRAF V600E or RAS p.Q61R was scored positive when tumor cells displayed a detectable granular cytoplasmic staining. The staining intensity was moderate to strong and relatively homogeneous (Figures 3 and 4). Ameloblastoma patients, positive for the mutation by IHC were also positive for the mutation as analyzed by genetic methods (Figure 1 and Appendix/Tables 1 and 2). The only exceptions were three *BRAF* V600E mutation positive AMs (cases 6, 34 and 36), which were negative with IHC, most likely because the stainings were done on decalcified tissue using formic acid, which is known to hinder detection of BRAF V600E by IHC.

Survival Analysis

A statistically significant result (p-value 0.0003) was obtained when comparing recurrence-free survival of AM patients with (N=12) and without (N=7) *BRAF* V600E mutations (Figure 5). The other Kaplan-Meier analyses did not yield statistically significant p-values due to the small number of patients (Appendix/Figure 3). The empirical odds of recurrence of *BRAF* wild-type tumors within the AM group were twice as high as in the *BRAF* V600E group of AMs (CI 0.17- 24.1, p-value 0.2).

Discussion

A number of unanswered questions persist regarding the diagnosis and treatment of UAM. Clinically, most patients are in their 2^{nd}–3^{rd} decade of life at the time of initial diagnosis. The majority of lesions develop in a dentigerous relationship to an unerupted tooth. They are unilocular on imaging but diagnosis is dependent on histopathological examination. This introduces another set of problems. Initial biopsy may include only the most accessible, cystic wall in which classical features of ameloblastoma may be absent. Such cases may include AM, diagnosed only after conservative excision. Most of the present UAM cases and many AMs were initially treated this way (Appendix/Tables 1 and 2). Extensive sampling was performed in all UAM cases to exclude conventional AM.

In the present study, 90% (35/39) of UAMs developed in the mandible, which is in line with a previously reported Chinese cohort (91%, 30/33) (Li et al. 2000). The reason for the predilection of both UAM and AM for the mandible, as well as the differences in the prevalence of mutations found between the mandible and maxilla is not known, but it is most likely linked to the differences in the expression of homeobox genes. These, such as *DLX* and *MSX,* are important transcription factors regulating the patterning of teeth, and their expression patterns differ in the upper and lower jaws (Thomas and Sharpe 1998).

The mean age at the primary diagnosis of UAM in the present study was 22.8 years in the case of association with an impacted tooth and 29.9 years without. In contrast, the mean age of the AM patients was 39.4 years. These findings coincide with previous data and the concept that the mean age of UAM patients is generally lower when the neoplasm is in

dentigerous relationship to an unerupted tooth, most often the third molar, than without impaction (Vered et at. 2017). The reason for this is unknown, but it is most likely linked to the origin of ameloblastoma from dental lamina (Heikinheimo et al. 2015).

~~The average time-to-first recurrence of UAMs was 6.1 years, comparable to the 7 years reported in the Chinese cohort (Li et al. 2000). Fourty four persent (14/32) of the UAMs recurred. All except one were enucleated. The average time-to-first recurrence of AMs was 7.8 years. Sixty seven percent (14/21) of the AMs recurred. Half of the primary AMs were treated by enucleation and half by resection. The first AM recurrence following enucleation was detected after an average of 6.2 years and among those treated by resection after 8.6 years. Taken together, these data highlight that, to ensure adequate management, long-term follow-up is also important for UAM.~~

In a recent study 62.5% of mandibular UAMs were reported to show *BRAF* V600E mutations as detected by TaqMan qPCR and Sanger sequencing (Pereira et al. 2016). In the present study, 94% of the mandibular UAMs revealed *BRAF* V600E mutations. In one *BRAF* wild-type mandibular tumor a SMO p.L412F mutation was identified. ~~This was an intraluminal UAM in association with an impacted tooth in an elderly patient.~~ Interestingly, Sweeney et al. 2014 reported *SMO* mutation in one mandibular and in ten maxillary AMs, and Brown et al. 2014 in two mandibular and in two maxillary AMs with or without a MAP-kinase mutation. SMO p. L412F is a well-known pathogenic mutation that seems to be functionally equivalent across different tumor types (Cosmic database: Mutation ID COSM2160377).

It is of interest that the UAMs seemed to be relatively uniformly positive for the *BRAF* V600E mutations, also associated with other benign and premalignant conditions (Kato et al. 2016), whereas a few AMs also harbored other mutations. Among the *BRAF* wild-type AM cases, two harbored NRAS p.Q61R, two HRAS p.Q61R and two FGFR2 p.C383R mutations. These hotspot mutations are commonly activated in various human malignancies and have also been reported in AM (Brown et al. 2014; Holderfield et al. 2014; Sweeney et al. 2014). The results of the present study show that the mutation profile of UAM appears to be more homogenous than that of the AM, in which other MAP-kinase-related mutations such as *RAS* and *FGFR2* are also found.

BRAF wild-type AMs have been reported to be more common in the maxilla (Brown et al. 2014, Sweeney et al. 2014) and relapse earlier than the *BRAF* mutated ones (Brown et al.

2014). In this study, a statistically significant association with an increased rate of recurrence (p-value 0.0003) was found in the wild-type AM group compared to the *BRAF* mutated group, as reported by Brown et al. 2014. We further evaluated the results using an odds ratio. The empirical odds ratio (2.0) showed that odds of recurrence in the BRAF wild-type group are twice as high as in the AM group, but due to the small number of patients (N=19), the p-value was not in the range of statistical significance. More ameloblastoma cases with sufficient follow-up data are needed to better illustrate the potential difference between the *BRAF* mutation status and recurrence in UAM and AM.

In our analyses, the *BRAF* V600E, *RAS*, *FGFR2* and *SMO* were mutually exclusive in ameloblastoma. However, it has been reported in other diseases such as in squamous cell carcinoma of the head and neck that mutual exclusivity as well as co-occurrence of mutations may define a subgroup of the disease (The Cancer Genome Atlas Network. 2015; Leemans et al. 2018). It is therefore tempting to hypothesize similar heterogeneity in AM, and a comprehensive analysis of the mutational spectrum could be of interest in UAM as well as in conventional AM.

In the present study, VE1 immunochemistry and molecular detection of the *BRAF* V600E mutations showed a high agreement in both the UAM and AM groups. Detection of the BRAF V600E mutation by VE1 immunohistochemistry has been reported to be 100 % sensitive and specific in colorectal cancer and 100% sensitive and 96.8% specific in melanoma (Thiel et al. 2013 and 2015), suggesting that VE1 immunochemistry performed on undecalcified tissue sections may be a valid surrogate for *BRAF* V600E genetic testing.

The morphologic classification of UAM is under debate and we were hoping to add some clarity to it based on the mutations occurring in each subtype. Although this is the biggest cohort to analyse mutations in UAM so far, the small number of samples precluded the statistical analyses of the different mutations with histological subtypes. Moreover, analyses of copy number alterations and epigenetic changes in ameloblastoma will require further experimentation with a larger dataset to strengthen the impact of BRAF V600E in this disease.

~~Associations with follow-up data demonstrated that 83% (10/12) of recurred UAMs featured *BRAF* V600E mutations with only a single case being negative (8%, 1/12). In contrast, 57% (8/14) of recurred AMs were *BRAF* V600E positive compared with 43 (6/14) wild-type tumors.~~

Conclusion

Our study demonstrates that *BRAF* V600E is the most common mutation in all three subtypes of UAM; luminal, intraluminal and mural. We also show that *BRAF* V600E is slightly more common in UAM (94%) than in AM (74%) and that *RAS* and *FGFR2* mutations are found in AM but not in UAM. It may be concluded, that dysregulation of the MAP- kinase pathway plays a critical role in the pathogenesis of both UAM and AM. It could therefore be argued that UAM and AM are part of a genetic as well as histomorphologic spectrum of the same odontogenic neoplasm. In light of the diagnostic and prognostic impacts of the mutation status, *BRAF* V600E analysis could be considered in routine ameloblastoma diagnostics.

Author Contributions

K. Heikinheimo contributed to the study design, data acquisition, analysis, and interpretation, and drafted the manuscript. J-M Huhtala, A Thiel, K. J. Kurppa, M. Kovac, K. Elenius, A. Ristimäki, D. Baumhoer and P.R. Morgan contributed to the study design, data acquisition, analysis, and interpretation, and critically revised the manuscript. C. Kragelund, G. Warfvinge, and H. Dawson contributed to data acquisition, diagnostics, and interpretation, and critically revised the manuscript. H. Heikinheimo performed the Kaplan-Mayer analyses and interpretation, and drafted and critically revised the manuscript. All authors gave their final approval and agree to be accountable for all aspects of the work.

Acknowledgments

The skillful technical assistance of Ms Mariia Valkama (Institute of Dentistry, University of Turku) and Ms Merja Haukka (University of Helsinki) is gratefully acknowledged. We are grateful to Mr Juha Kettunen for the data acquisition and design of the tables and figures. The work was financially supported by the Maritza and Reino Salonen Foundation. The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

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Figure legends

Figure 1. Oncogenic mutations in unicystic ameloblastoma (UAM) and conventional ameloblastoma (AM) samples. Interactive presentation available in www.kristiinaheikinheimo.fi/uamam. By clicking each case symbol, the patient data is shown.

A. Mutations in association with the clinical parameters in UAM and AM including the age of the patient, location in mandible versus maxilla and the connection of UAMs with an unerupted tooth. The mean and median ages at primary diagnosis of the UAM and AM patients are shown. In two UAMs and one AM patient the age at primary diagnosis was not available.

B. Thirty UAM samples were *BRAF* V600E positive as detected by genotyping. One UAM was SMO p.L412F positive as detected by targeted next-generation and Sanger sequencing. Three UAM samples were wild type and five samples could not be processed because of the poor quality or insufficient amount of the DNA. Twenty-eight AM samples were BRAF V600E positive, four RAS Q61R positive (out of which two were HRAS and two NRAS), and two FGFR2 p.C382R positive by Sanger sequencing and next-generation sequencing. In six AM cases no mutations were detected.

Figure 2. Sanger sequencing showing the mutated nucleotide responsible for the BRAF c.1799T>A (p.V600E), SMO c.1234C>T (p.L412F), FGFR2 c.1144T<C (p.C382R), NRAS c.182A>G (p.Q61R) substitution (arrow).

Figure 3. Immunohistochemical staining in unicystic ameloblastomas using BRAF V600E mutation-specific monoclonal antibody. A. Luminal type, B. Intraluminal type, C. Mural type. In all variants intracellular staining is uniform throughout the epithelium.

Figure 4. Immunohistochemical staining in ameloblastomas using NRAS Q61R mutation-specific rabbit monoclonal antibody. A. Conventional ameloblastoma (case 19) with staining of all cells in the epithelial follicles, B. Unicystic ameloblastoma, typically non-staining. Positive control tissues: C. Rectal carcinoma. D. Melanoma.

Figure 5. Recurrence-free survival probability of *BRAF* V600E positive (N=12) and negative (N=7) conventional ameloblastomas as a function of years elapsed after last treatment.

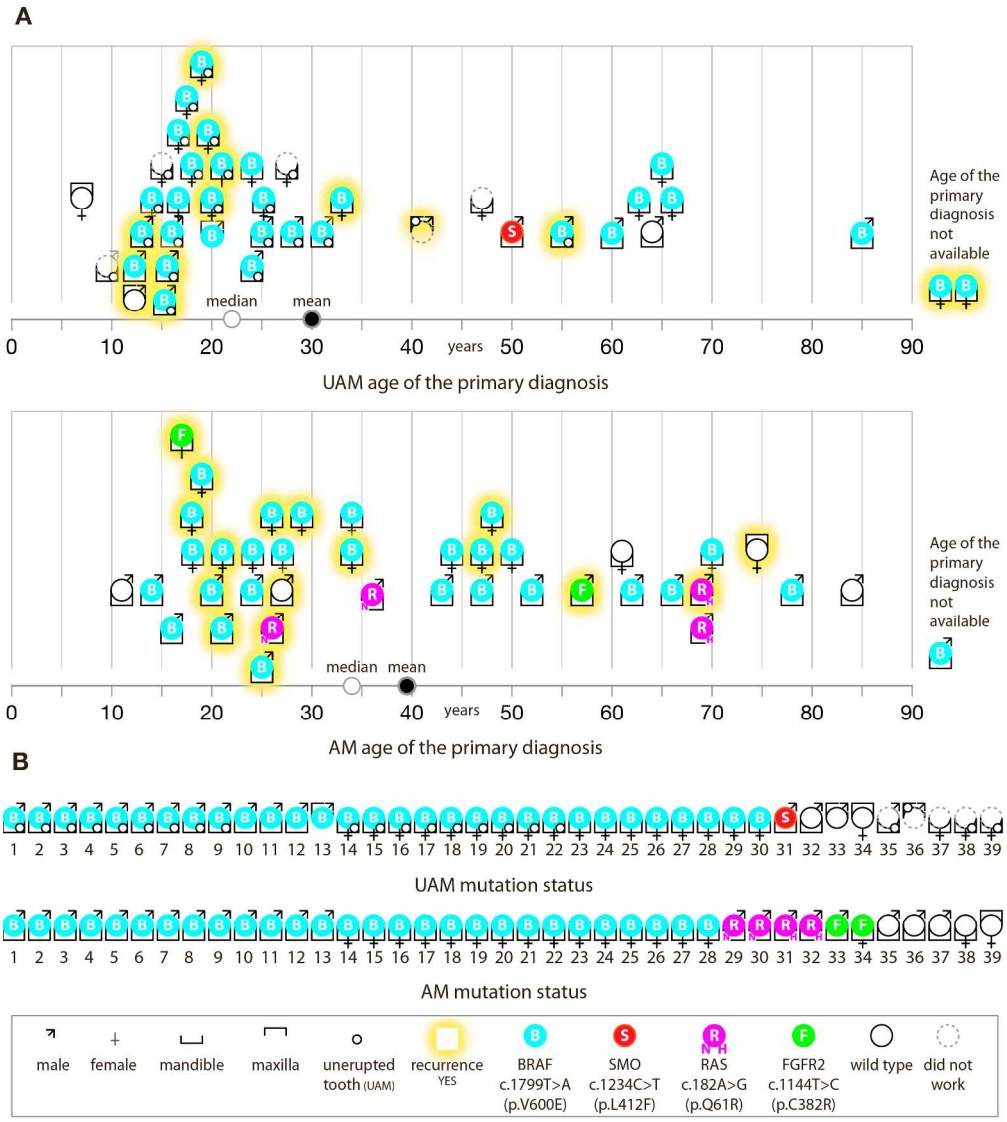

Figure 1. Oncogenic mutations in unicystic ameloblastoma (UAM) and conventional ameloblastoma (AM) samples. Interactive presentation available in www.kristiinaheikinheimo.fi/uamam. By clicking each case symbol, the patient data is shown.‼ ┼ A. Mutations in association with the clinical parameters in UAM and

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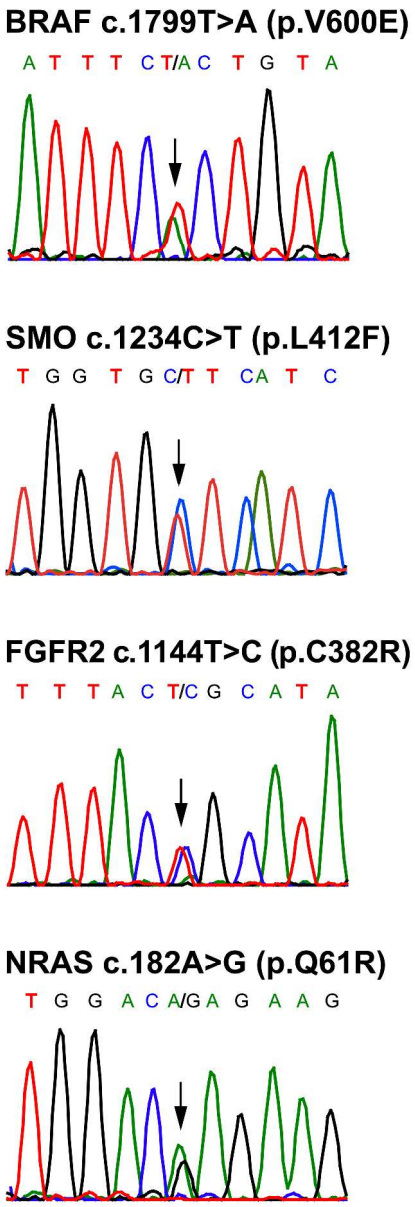

Figure 2. Sanger sequencing showing the mutated nucleotide responsible for the BRAF c.1799T>A (arrow).

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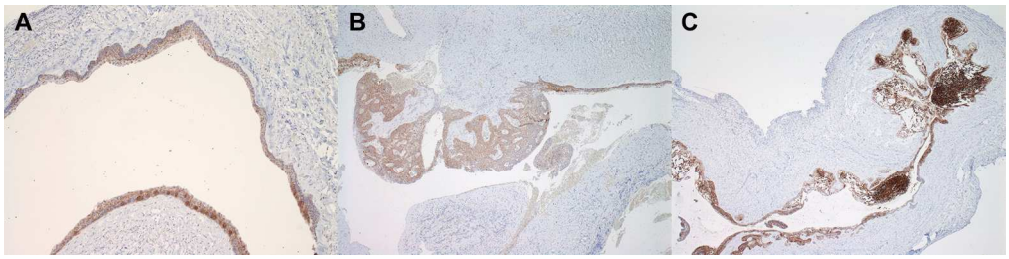

Figure 3. Immunohistochemical staining in unicystic ameloblastomas using BRAF V600E mutation-specific monoclonal antibody. A. Luminal type, B. Intraluminal type, C. Mural type. In all variants intracellular staining is uniform throughout the epithelium.

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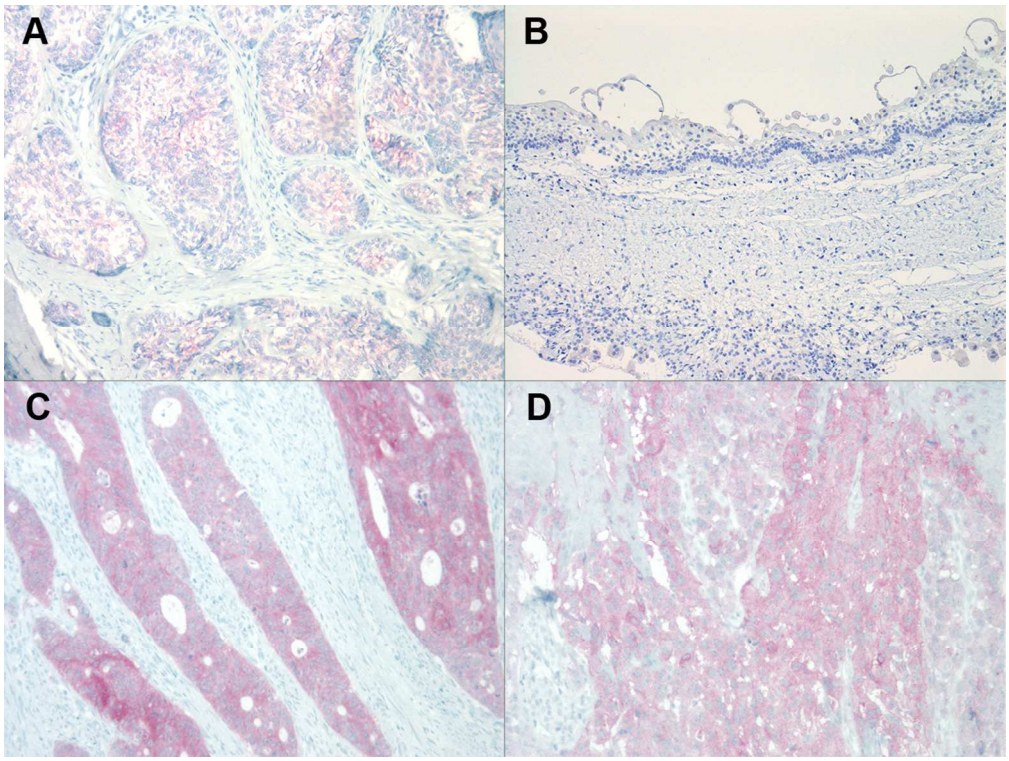

Figure 4. Immunohistochemical staining in ameloblastomas using NRAS Q61R mutation-specific rabbit monoclonal antibody. A. Conventional ameloblastoma (case 19) with staining of all cells in the epithelial follicles, B. Unicystic ameloblastoma, typically non-
Melanoma.

115x86mm (300 x 300 DPI)

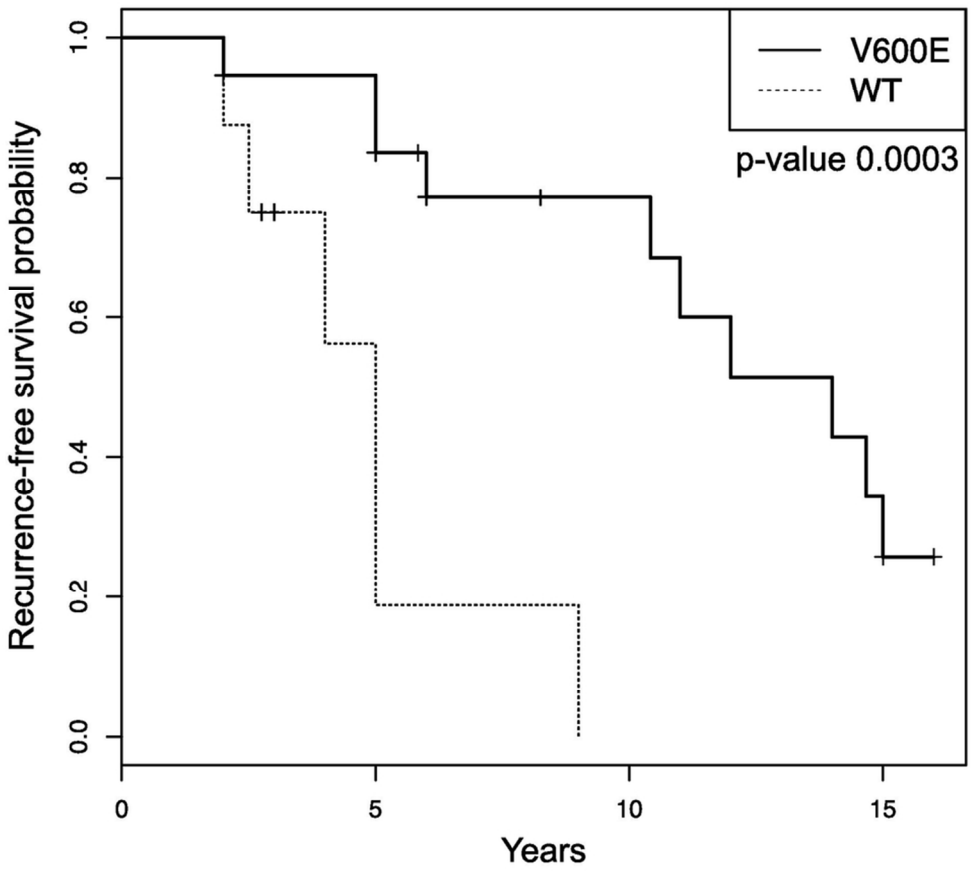

Figure 5. Recurrence-free survival probability of BRAF V600E positive (N=12) and negative (N=7) conventional ameloblastomas as a function of years elapsed after last treatment.

83x83mm (300 x 300 DPI)

The mutational profile of unicystic ameloblastoma \ Appendix

Short title: Oncogenic mutations in unicystic ameloblastoma

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Appendix/Table 1. Clinico-pathological information and mutation status of the unicystic ameloblastomas (N=39).

Case	Age	Sex	Ethnicity	Location		Treatment	Histological type			NGS
9	20	M		Maxilla	NO	Primary	Enucleation	Luminal	V600E	Positive	N.A.
13	7	F		Maxilla	NO	Primary	Enucleation	Luminal	WT	Negative	Negative
15	14	F		Mandible	YES	Primary	Enucleation	Mural	V600E	Positive	N.A.
17	50	M	Caucasian	Mandible	YES	Primary	Resection	Intraluminal	WT	Negative	

Abbreviations: F female; M male; NA not available, N.A. not analyzed; WT wild type; G genotyping; S Sanger sequencing; NGS next generation sequencing; Rec recurrence.

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Appendix/Table 2. Clinico-pathological information and mutation status of the conventional ameloblastomas (N=39).

Case	Age	Sex	Ethnicity	Location	Primary Rec	Treatment	Histological type	BRAF status	Geno-typing Sanger	BRAF V600E IHC	status	status	status	NRAS/ HRAS IHC	NGS
16	61	F	N.A.	Mandible	Primary	Enucleation		WT	S	Negative	WT	WT	WT	Negative	Negative
20	44	F	Black African	Mandible	Primary			V600E	S	Positive	WT	WT	WT	Negative	N.A.
22	46	F	Afro Caribbean	Mandible	1 Rec	Resection	Plexiform	V600E	S	N.A.	WT	WT	WT	Negative	N.A.
27	35	M	Caucasian	Mandible	1 Rec		Follicular	V600E	G	Positive	N.A.	N.A.	N.A.	Negative	N.A.
33	37	F	Caucasian	Mandible	1 Rec	Resection		V600E	G	Positive	N.A.	N.A.	N.A.	Negative	N.A.

Abbreviations: F female; M male; NA not available; N.A. not analyzed; WT wild type; G genotyping; S Sanger sequencing; NGS next generation sequencing; Rec recurrence.

Appendix/Table 3. Follow-up information of the unicystic ameloblastoma patients (N=39).

	Follow up in months						
Case	Age at primary detection	1st recurrence time after	2nd recurrence time after	3rd recurrence time after	4th recurrence time after	Follow up from primary detection	Recurrence at the end of the follow-
1	85					48	NO
2	60					25	NO
3	41	12				192	NO
4	24						
5	27						
6	28					72	NO
7	10						
8	31						
9	20					60	NO
10	47						
11	15						
12	12	24				84	NO
13	7					96	NO
14	18					24	NO
15	14					60	NO
16	65					84	NO
17	50					10	NO
18	17					7	NO
19	17					16	NO
20	25					17	NO
21	24					24	NO

22	NA	N.A.	N.A.			46	NO
23	NA			46		92	YES
24	66					47	NO
25	12	265				337	NO
26	19	46	90			123	NO
27	22					79	YES
28	17					99	NO
29	64					99	NO
30	33	19				121	NO
31	25					106	NO
32	63					130	NO
33	16					153	NO
34	55	44				170	NO
35	20	16	79	103	199	280	NO
36	19					177	YES
37	15	10				180	NO
38	15					182	YES
39	13	19				19	YES

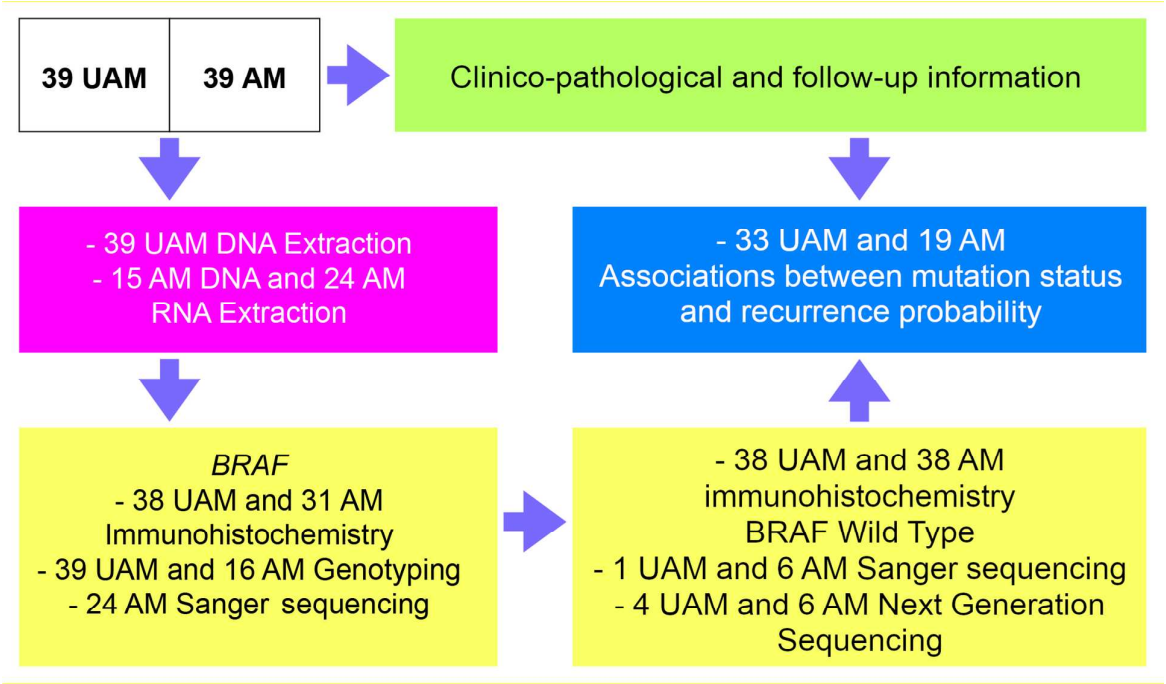
Abbreviations: NA not available.

Appendix/Table 4. Follow-up information of the conventional ameloblastoma patients (N=39).

	Follow up in months				
Case	Age at primary detection (years)	1st recurrence time after primary detection	2nd recurrence time after primary	Follow up from primary detection	Recurrence at the end of the follow- up period
1	66				
2	70				
3	57	48		48	YES
4	27				
5	24				
6	50				
7	36				
8	47				
9	27	60		60	YES
10	25	NA	252	252	YES
11	14				
12	19	176		216	NO
13	84				
14	18				
15	16			72	NO
16	61				
17	69	24		24	YES
18	69				
19	43			192	NO
20	44			60	NO
21	20	NA	24	24	YES

22	34	144		144	YES
23	62				
24	26	60		60	YES
25	29	60		159	NO
26	11			33	NO
27	21	168		360	NO
28	52				
29	78				
30	NA				
31	21	180	305	377	NO
32	18	60		180	NO
33	26	132		132	YES
34	34				
35	65	108		144	NO
36	48	NA	276	276	YES
37	24				
38	47	72		96	NO
39	17	30		30	YES

Abbreviations: NA not available



Appendix/Figure 1. Flow chart of the study. Clinico-pathological and follow-up information were collected from 39 UAM and 39 AM patients. DNA was extracted from 39 UAM and 15 AM FFPE blocks and RNA from 24 fresh frozen AM tissues. *BRAF* V600E mutations were first analyzed by VE1 immunohistochemistry (IHC) from 38 UAMs and 31AMs, by genotyping from 39 UAMs and 16 AMs and by Sanger sequencing from 24 AMs. Ameloblastomas where thereafter analyzed by RAS p.Q61R IHC. Four AMs were RAS IHC positive. The presence of RAS mutations in these four AMs were confirmed by Sanger sequencing. Two BRAF wild-type UAMs underwent targeted next-generation sequencing (NGS). NGS was also performed for four wild-type UAMs and six wild-type AMs. One UAM harbored SMO p.L412F mutation and two AMs FGFR2 p.C382R mutations, which were confirmed by Sanger sequencing. Finally, Kaplan-Meier-analyses were performed on 33 UAMs and 19 AMs, which had follow-up data available.

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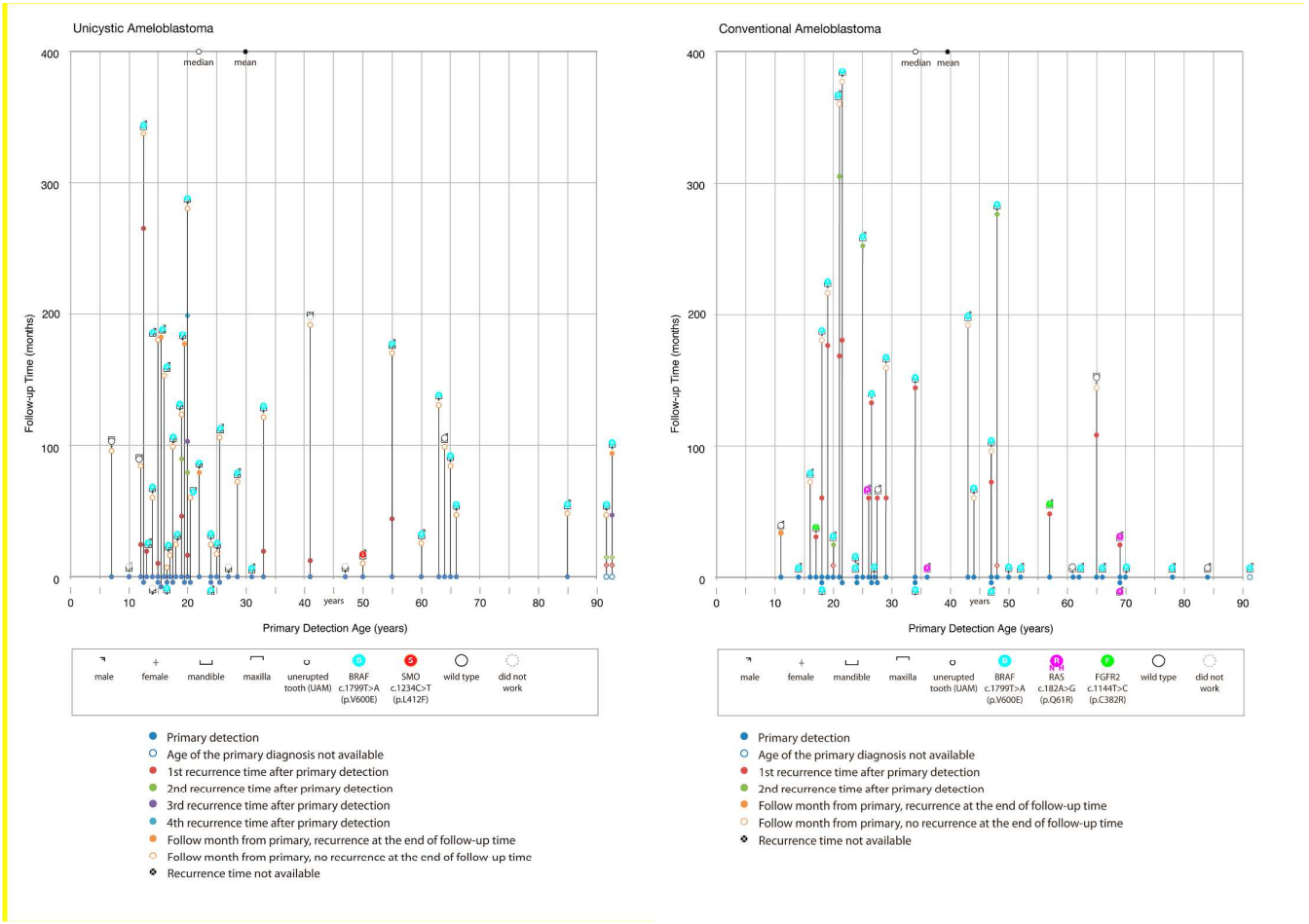
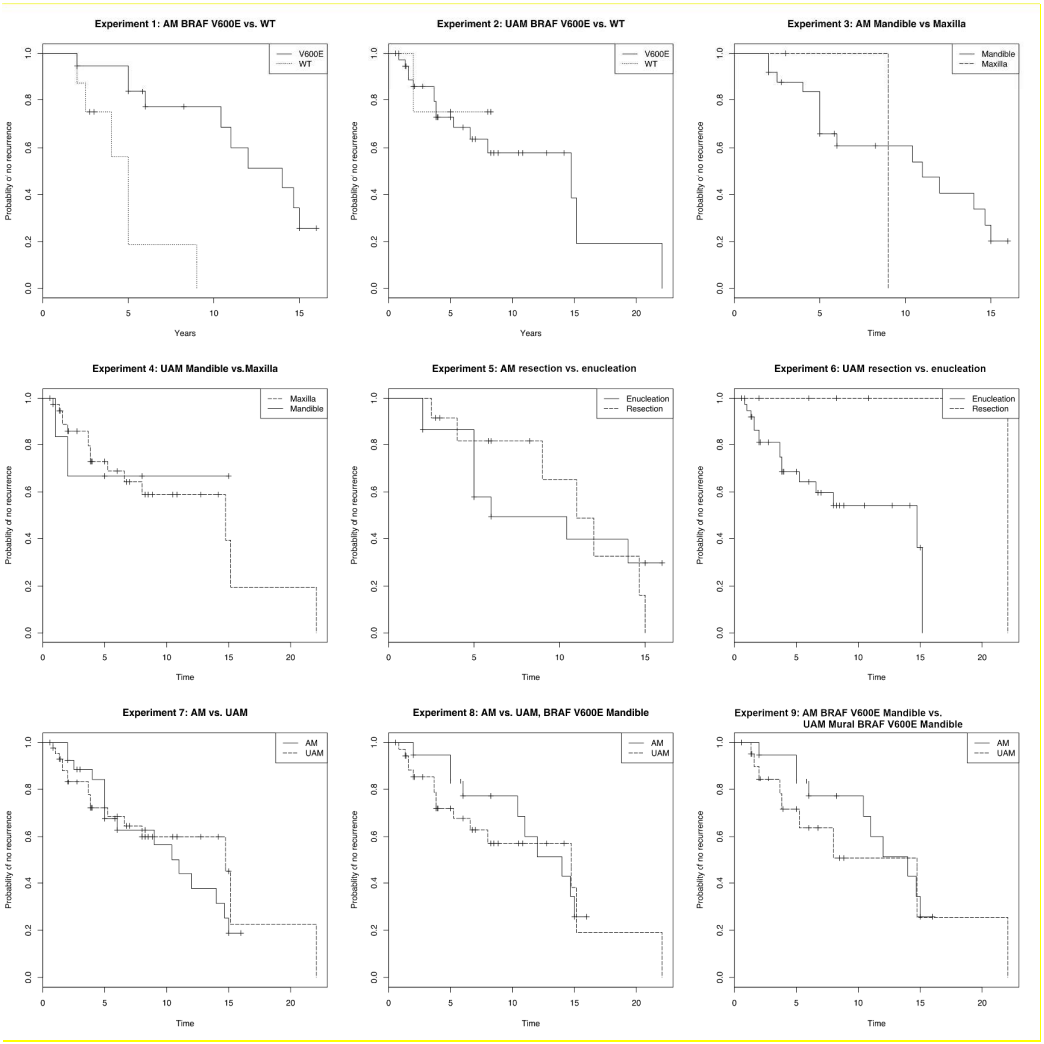


Appendix/Figure 2. Graph visualizing the follow up of each ameloblastoma patient together with the mutation status. The X-axis shows the age of the patient at the time of primary detection and the Y-axis shows the follow-up time-line. 33/39 UAM and 21/39 AM cases had follow-up information available. The average follow-up time for the UAMs was 8.2 years and for the AMs and 11,7 years. 14/39 UAMs and 17/21 AMs had one or multiple recurrences, which are marked in the time-lines with colored circles. At the end of the follow-up period, patients without a reported recurrence are marked with a yellow open circle.



Appendix/Figure 3. Charts from Kaplan-Meier analyses for comparing recurrence-free survival between UAMs (N=33) and AMs (N=19) as groups and as sub-groups. The two groups were compared in relation to *BRAF* V600V vs. wild-type (charts 1-2), mandible vs. maxilla (charts 3-4), and resection vs. enucleation (charts 5-6). AM and UAM groups were compared (chart 7), the mandibular *BRAF* V600E

positive UAM and AM groups (chart 8), and the mandibular AM *BRAF* V600E positive group against the mandibular mural *BRAF* V600E positive UAMs (chart 9). Experiment 1 gave the only statistically significant result (p-value: 0.000346).