Prediction of Ameloblastoma Aggresivity with Clinicopathological Examination, $BRAF^{V600E}$ and Ki-67 Genes

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Abstract

Cases of ameloblastoma in young patients with recurrences are increasingly found. It is known that the clinicopathology of aggressive ameloblastoma is associated with mutations in the BRAFV600E and Ki-67 genes. A new strategy is needed for pathogenesis prediction based on demographics, clinical features, and biological behavior of ameloblastoma.

This study aims to analyze and predict the scoring of the relationship of demographic factors, clinical characteristics, and biological behavior through BRAFV600E and Ki-67 gene expressions with the aggressivity of ameloblastoma in the population in Indonesia.

This study was conducted retrospectively (2014-2020). Analysis was performed on demographic status; local status: impacted tooth, root resorption, tumor location, tumor size, duration of the tumors,lymph node enlargement, the direction of the tumor invasion; radiologic results; postoperative diagnosis; clinicopathological subtype; operation management; recurrence; and BRAFV600E and Ki-67 gene expressions.

A total of 45 test samples consisted of 15 males (33.3%) and 30 females (66.7%) with a mean age of 34 years old from the range of 6-65 years old. From the observation of the Odd's ratio value in multivariate analysis, the risk of a person with a BRAFV600E expression ratio: Ki-67 expression ≥ 0.32 to develop aggressive ameloblastoma was 5.62 times greater than someone with a BRAFV600E expression: Ki-67 expression level < 0.32. In addition, the risk of someone who had developed a tumor for ≥12 months was 2.72 times greater than that of < 12 months to develop aggressive ameloblastoma. The risk of a person who had an impacted tooth to develop an aggressive ameloblastoma was 0.857 times lower than that of a person who did not have an impacted tooth.

This study showed that examination of BRAFV600E immunoexpression, Ki-67 expression, and BRAFV600E gene mutation could predict the aggressivity of ameloblastoma.

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Introduction

According to the WHO consensus in 2017, Ameloblastoma is a benign epithelial odontogenic intraosseous neoplasia characterized by expansion, infiltration, invasion, and a tendency to recur locally.[1] This tumor is classified as benign with the underlying cells from the epithelium involved in tooth formation, remnants of the enamel organ, epithelial cell remnants of Malassez, of Serres, of Hertwig sheath, and epithelial lining of odontogenic cysts (the most common are dentigerous cysts), but it rarely grows in the soft tissues of the gingiva.[2] The trigger of the neoplastic transformation of the tooth-forming epithelial remnants is still unknown.[2,3] The incidence of Ameloblastoma is reported to be around 1% of all tumors in the oral cavity or about 9-11% of all odontogenic tumors.[4] The global incidence of Ameloblastoma is 0, 5 cases per million population, being more common in Asia and Africa, comprising 14% of all tumor and cystic lesions occurring in the

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maxilla and mandible.[4] About 80% of ameloblastomas arise in the mandible, mainly in the region of the lower third molars, while the remaining 20% are in the maxilla.[4]

Cases of Ameloblastoma in young patients were increasing in the oral and maxillofacial surgery clinic of Dr. Cipto Mangunkusumo Hospital (RSCM). Patients often did not know and were unaware of this tumor due to the absence of pain during its growth and development. The growth and enlargement of this odontogenic tumor are slow but aggressive. Clinicopathological parameters of its aggressivity can be seen from the size of the tumor, root resorptions around the tumor, the presence of impacted tooth around the tumor, tumor invasion to surrounding tissues, the duration of the tumor, and the presence of regional metastases to the lymph nodes in the colli region. Aggressivity and recurrences in ameloblastoma are like two sides of an inseparable coin, where recurrence occurs due to the aggressivity of the tumor that infiltrates the hard tissue to the surrounding soft tissue. Thus, inadequate surgical removal of the tumor increases the risk of recurrences. Recurring ameloblastoma poses a challenge to find new modalities and therapeutic strategies for clinicians and researchers. Mandibulectomy of ameloblastoma with 1–1.5 cm extension to the healthy bone is adequate to manage ameloblastoma. However, surgeons must pay attention to the safety margin during soft- tissue excision, especially in the case of aggressive acanthomatous ameloblastoma.

To date, several genetic mutations in Ameloblastoma have been reported. The most common gene found mutated in Ameloblastoma is $BRAF^{V600E}$, which varies between 43%-97%.[5,6] Approximately 90% of BRAF mutations are transversion of thymine to adenine at exon 15 of nucleotide 1799 (T1799A), which results in the substitution of valine into acid. Glutamate at position 600 (BRAFV600E) results in excessive BRAF activation.[7] The $BRAF^{V600E}$ mutation induces the Mitogenic-Activated Protein Kinase (MAPK) pathway that plays a role in tumorigenesis.[7,8] In addition, Ki-67 protein, which plays a role in mitosis and cell cycle, has been widely used as a marker of human tumor cell proliferation because abnormal cell proliferation is a hallmark of tumor growth.[9] However, to date, no study has shown the association between mutations in the BRAF and Ki-67 genes on the aggressivity and recurrence of ameloblastoma and associating it with clinicopathological conditions.

The difficulty of making a histopathological diagnosis of ameloblastoma encourages the search for prognostic and predictive factors that play a role in aggressive growth of ameloblastoma to prevent recurrences and metastasis. There are several standard methods of DNA-based examination, such as DNA direct sequencing, pyrosequencing, and PCR/real-time polymerase chain reaction in identifying BRAF gene mutations in ameloblastoma. This method has a high level of accuracy. However, they pose several challenges: acquiring adequate DNA concentration and purity is difficult, they need specific equipment and reagents which are not always available, they need trained professionals, the process is long and expensive. Immunohistochemical (IHC) examination using primary monoclonal antibody anti-$BRAF^{V600E}$ (VE1) can also be used to identify BRAF mutations in various malignancies, including ameloblastoma.[6] This method has good sensitivity and specificity in detecting BRAF gene mutations of 98%-100%.[6] The main advantages of IHC examination compared to other molecular biology techniques are faster results and low cost.

Based on the background of the problems above, this study aims to find the relationship between various clinicopathological parameters of aggressivity and recurrence of ameloblastoma with changes in the expression of the $BRAF^{V600E}$ gene and the Ki-67 gene using the IHC method, as well as the possibility of establishing a diagnostic, predictive model for patients with ameloblastoma in RSCM.

Materials and methods

Descriptive study

Evaluation of the clinical characteristics of the subjects included age, sex, occupation, education, ethnicity, tumor location, and extension to the lymph gland of the colli regio. Meanwhile, anatomic pathological analysis was performed on ameloblastoma biopsy specimens with Hematoxylin and Eosin (HE) staining to determine ameloblastoma subtypes. The objects to be examined are ameloblastoma odontogenic tumor cells taken during surgical management. Various scoring factors will be analyzed as

variables determining the degree of differentiation, including: number of mitoses, keratinization and nuclear polymorphism, as well as variables determining the degree of aggressivity and recurrences from the aspect of tumor cell population. In addition, in order to obtain the degree of aggressivity, scoring factors will be analyzed in terms of the relationship between tumor cells and the host, namely invasion patterns and lymphoplasmocytic infiltration. The results will be tested for sensitivity and specificity with anatomic pathologic diagnosis as the reference standard. This study is focused on diagnostic studies.

This study is a cross-sectional study with the aim of finding causal relationships between causal factors that play a role in the growth and development of ameloblastoma, in this case analyzing the aggressivity factors involved in expansion, mass infiltration into surrounding soft tissues, invasion into the lymph gland in the colli, and recurrence in the correlation between the presence of variations in mutations of the $BRAF^{V600E}$ and Ki67 genes, with anatomical pathological parameters in ameloblastoma.

Cross-sectional study

A cross-sectional study with a retrospective, non-randomized approach was conducted on surgical specimens from patients with ameloblastoma. These operative specimens were divided into different types of ameloblastoma. This research design aims to answer the research question, which is to obtain the strength of the correlation (r) between various ameloblastomas and the specific and overall aggressivity of ameloblastoma against the expression levels of the $BRAF^{V600E}$ and Ki-67 genes. Variables were determined based on pathologic anatomical evaluation and IHC results. Correlations were determined by bivariate analysis using regression equations. Multivariate logistic regression analysis was used to analyze predictors. Confounding variables were patient's age, sex, tumor location, occupation, education, and race.

Research Subjects

The research subjects were taken from paraffin blocks made in the Anatomical Pathology laboratory of RSCM / FKUI from patients with a histopathological diagnosis of ameloblastoma who had undergone surgery in 2017 – October 2021. The sample collection process was carried out by consecutive sampling which met the inclusion criteria of the study. Samples that did not meet the criteria such as unavailable biopsy results, damaged paraffin blocks, and incomplete medical record data were not included in the study sample.

Data Processing and Analysis Technique

The data obtained were coded, tabulated, statistically calculated with SPSS 26 software with a significance limit $p < 0.05$ and 95% CI. Univariate analysis was performed to assess the distribution of demographic variables, oral hygiene status, $BRAF^{V600E}$ and Ki-67 expression, $BRAF^{V600E}$ mutation level and clinical examination of ameloblastoma. Bivariate analysis was performed to assess the effect of oral hygiene on ameloblastoma aggressivity, $BRAF^{V600E}$ expression on ameloblastoma aggressivity, Ki-67 expression on ameloblastoma aggressivity, $BRAF^{V600E}$ mutation on ameloblastoma aggressivity.

Multivariate analysis was performed to establish $BRAF^{V600E}$ expression, Ki-67 expression, and $BRAF^{V600E}$ mutation as indicators of aggressive ameloblastoma, and to establish a prediction index for the ratio of $BRAF^{V600E}$ and Ki-67 expression by clinical examination of aggressive ameloblastoma.

Assessment of Ki-67 immunopositive cells was performed by assigning thyroid tissue as the positive control, then Ki-67 immunostaining was evaluated quantitatively. The average number of positively stained nuclei was counted in each stained section in 10 high-power microscopic fields (x400). The final IHC score was calculated by summing all positively stained nuclei in 10 high-power fields and dividing by 10. The results were calculated using Cohen's kappa test for intraobserver reliability with a result of 0.767 (substantial agreement) and interclass correlation coefficient (ICC) test for interobserver reliability with a result of 0.876 (excellent agreement).

- Weak (+) < 5 positive epithelial cells
- Medium (++) 6-10 positive epithelial cells
- Strong (+++) 11-20 positive epithelial cells
- Very Strong (++++) > 21 positive epithelial cells

Analysis of immunoreactive $BRAF^{V600E}$ IHC was assessed independently by two people. Cytoplasmic staining of neoplastic epithelium was considered positive for $BRAF^{V600E}$ expression. Staining intensity and proportion were assessed by slight modification of the

criteria provided by Reiner et al and Barnes et al.[10,11] The average intensity of the entire tissue section was evaluated and scored as 0 (no staining), 1 (visible at low power magnification, x 10) and 3 (visible on scanner view, x 4). The total proportion of positive cell staining at any intensity was assessed by randomly screening five fields per tissue section as 0 (no cell staining), 1 (when 1%-5% of cells were stained) and 4 (when >50% of cells were stained). The intensity results and proportion of positively staining cells were combined to give a quick score as follows; 2-3 points = low, 4-5 points = medium, and 6-7 points = high positive for $BRAF^{V600E}$ immunoexpression.

The data in this study were obtained from patients who sought treatment at the oral and maxillofacial surgery clinic of RSCM whose tissues were archived in the Anatomical Pathology section of RSCM / Faculty of Medicine Universitas Indonesia (FKUI). The research was conducted by keeping the subject's data confidential. This research had obtained ethical approva from the Faculty of Dentistry Universitas Indonesia (FKGUI) Ethics Commission Number: 2/Ethical Approval/FKGUI/III/2021 with Protocol Number: 070010221.

Results

Descriptive Analysis

In this study, 90 samples of ameloblastoma were obtained, but only 55 samples met the inclusion criteria. Furthermore, by using the consecutive sampling method, 45 test samples were obtained. PCR examination were performed only on 24 samples, consisting of 12 primary status samples and 12 recurrence samples. The sample data for this study are shown in Table 1, consisting of 15 males (33.3%) and 30 females (66.7%). The average age of ameloblastoma patients was 34 years old, with the overall average age of 6-65 years. Characteristics of age were < 34 years = 23 cases (51.1 %), and 34 = 22 cases (48.9%), whilst characteristics of races were Javanese = 20 cases (44.4%), Sundanese = 8 cases (17.8%), and others = 17 cases (37.8%).

The locations of the tumor were mandibular anterior tumor (C-C)= 9 cases (20.0%), mandibular anterior to the angle= 6 cases (13.3%), mandibular anterior-angulus-ascending ramus= 25 cases (55.6%), and maxilla= 5 cases (11,1%). Radiologically, 8 cases (17.8%) were unilocular ameloblastoma, while multilocular lesions were 37 cases (82.2%). Overall, based on histopathological examination with conventional HE staining in a total of 45 cases of ameloblastoma, there were 13 cases (28.9%) consisted of single subtype (follicular, plexiform, acanthomatous, or desmoplastic), whilst 29 cases (64.4%) were multiple subtype ameloblastoma (follicular + plexiform + acanthomatous + desmoplastic), and 3 cases (6.7%) were ameloblastic carcinoma. There were 25 cases (55.6%) of primary ameloblastoma and 20 cases (44.4%) of recurrent ones. The durations of the tumor were <12 months= 11 cases (24.4%) and ≥12 months = 34 cases (75.6%). This study also found the presence of impacted tooth within the tumor in 18 cases (40%), and without impacted tooth in 27 cases (60%). Meanwhile, patients with normal BMI were 23 cases (51.1%), obese patients were 13 cases (28.9%), and below normal cases were 9 subjects (20.0%). Five cases (11.1%) were treated conservatively and 40 cases (88.9%) radically/aggressively.

	Total	Mutation (%)	No Mutation (%)	OR	*p* value
Sex					
Male	24	10 (41.7)	2 (8.3)	1.67	1,000
Female		9 (37.5)	3 (12.5)		
Age					
< 34 years old	24	10 (41.7)	4 16.7)	3.60	0.358
≥ 34 years		9 (37.5)	1 (4,2)		
Location of the tumor in the jaw					
lower jaw	24	16 (66.7)	5 (20.8)		
Upper jaw		3 (12.5)	0 (0)	0.76	1,000
Tumor Location					
Anterior Jaw	24	4 (16.7)	2 (8.3)	2.50	0.568
Posterior Jaw		15 (62.5)	3 (12.5)		
Tumor Status					
Primary	24	8 (33.3)	4 (16.7)	5.50	0.317
Recurrence		11 (45.8)	1 (4,2)		
Subtype					
Single (Fol/Plex/Acant/Desmo)	24	7 (29.2)	3(12,5)	2.57	0.615
Multi (Fol + Plex + Acant + Desmo)		12 (50)	2 (8.3)		
Invasion to lymph nodes					
Yes	24	14 (58.3)	4 (16.7)	0.70	1,000
No		5 (20.8)	1 (4,2)		

Description: * Fisher's exact test, $p<0.05$ = significant

Table 1. Sociodemographic influence; sex, age, tumor location in the jaw, tumor status, ameloblastoma subtype, and invasion to the KGB. against the $BRAF^{V600E}$ mutation.

The results of the $BRAF^{V600E}$ IHC examination on 45 subjects showed 37 positive subjects (82.20%) and 8 negative subjects (17.8%). The results of the Ki-67 IHC examination on 45 subjects found 25 positive subjects (55.60%) and 20 negative subjects (44.4%). The results of the PCR examination of a total of 24 subjects showed that 19 subjects (79.2%) were positive and 5 subjects (20.8%) were negative.

The aggressivity characteristics of ameloblastoma (Table 2) were reported based on 4 categories: (i) tumor size: < 7 cm in 29 cases (64.4%) and ≥7 cm in 16 cases (25.6%); (ii) invasion to the cortical bone: lingual/palatal side in 17 cases (37.8%), to the labial/buccal side in 2 cases (4.4%), and in all directions in 26 cases (57.8%); (iii) root resorption of the involved teeth in the tumor in 29 cases (64.4%) and no resorption in 16 cases (35.6%); (iv) the presence of regional metastases in the lymph nodes in 35 cases (77.8%) and no metastases in 10 cases (22.2%).

		IHC	*BRAF*				*PCR*	*BRAF*		
Clinic Characteristics	**n**	**Positive (%)**	**Negative (%)**	**OR**	***p* value**	**n**	**Mutation (%)**	**Wild type (%)**	**OR**	***p* value**
Sex	45									
Male		14 (31.1)	1 (2,2)	4.26	0.236	24	10 (41.7)	2 (8.3)	1.67	1,000
Female		23 (51.1)	7 (15.6)				9 (37.5)	3 (12.5)		
Age	45									
< 34 yrs old		20 (44.4)	3 (6.7)	0.51	0.459	24	10 (41.7)	4 (16.7)	3.60	0.358
34 years old		17 (37.8)	5 (11,1)				9 (37.5)	1 (4,2)		
Tumor duration	45									
< 12 years old		8 (17.8)	3 (6.7)	2.17	0.382	24	5 (20.8)	2 (8.3)	1.87	0.608
12 years old		29 (64.4)	5 (11,1)				14 (58.3)	3 (12.5)		
Tumor size	45									
< 7 cm		20 (44.4)	5 (11.1)	1.42	0.716		9 (37.5)	4 (16.7)	4.44	0.327
>/= 7 cm		17(37,8)	3 (6.7)			24	10 (41.7)	1 (4,2)		
Tooth impaction	45									
Yes		15 (33.3)	3 (6.7)	1.14	1,000		7 (29.2)	1 (4,2)		
No		22 (48.9)	5 (11,1)			24	12 (50.0)	4 (16.7)	2.33	0.631
Tooth root resorption	45									
Yes		25(55,6)	4 (8,9)	2.08	0.427	24	13 (54.2)	4 (16.7)		1,000
No		12 (26.7)	4 (8,9)				6 (25.0)	1 (4,2)	0.542	
Enlargement in the KGB	45									
Yes		28 (62.2)	7(15,6)	0.44	0.661	24	14(58,3)	4(16,7)	0.700	1,000
There is none		9 (20.0)	1 (2,2)				5 (20.8)	1(4,2)		

Description: * Fisher's exact test, $p<0.05$ = significant

Table 2. The relationship between clinical characteristics and BRAF^{V600E} mutation status detected by two methods.

The proportion of BRAF^{V600E} and Ki-67 IHC results on the research characteristics of 45 subjects can be seen in Table 3. The ages of most subjects were <34 years where the highest number of positive BRAF^{V600E} was 20 subjects (44.4%) and negative was 3 subjects (6.7). %), while Ki-67 was positive in 14 subjects (33.3%) and negative in 8 subjects (17.8%). Female had positive BRAF^{V600E} with 23 subjects (51.1%) positive and 5 subjects (15.6%) negative, while the IHC Ki-67 results of 18 subjects (40.0%) were positive and 12 subjects (26.7%) were negative. For the ameloblastoma subtype, the highest number was in the mixed subtype with BRAF ^{V600E} positive in 24 subjects (53.3%) and negative in 5 subjects (11.1%), whilst Ki-67 was positive in 17 subjects (37.8%) and negative in 12 subjects (26.7%). The most frequent radiological findings were multilocular with 31 subjects (68.9%) BRAF ^{V600E} positive and 6 subjects (13.3%)BRAF ^{V600E} negative, whilst Ki-67 positive was 21 subjects (46.7%) and negative was 16 subjects (35 ,6%.)

Bivariate Analysis

Overall, the data found are presented in Table 1 with demographic distribution, including sex, age, histopathological subtype, tumor location, tumor size, radiological features, involvement of the regional lymph nodes, and BMI.

Table 1 shows that there is no statistically significant effect ($p < 0.05$) on the BRAF^{V600E} mutation on sex, age, tumor location, tumor status, ameloblastoma subtype, and invasion of surrounding soft tissue. This table also shows the most mutations in the mandible in 16 of 19 samples (66.7%) and the maxilla in 3 of 19 samples (12.5%), and 15 of 19 samples (62.5%) were in posterior, and 4 of 19 samples (16.7%) were in anterior. The mutations occurred mostly in recurrence case which were 11 out of 19 samples (45.8%), while primary status was 8 out of 19 samples (33.3%). The most mutated ameloblastoma subtypes were in mixed type, which were in 12 out of 19 samples (50%), and 7 out of 19 samples (29.2%) were in non-mixed ameloblastoma. Meanwhile, 14 out of 19 samples (58.3%) mutated ameloblastoma invaded the lymph nodes and 5 out of 19 samples (20.8%) did not.

Table 2 shows that there is no significant ($p > 0.05$) relationship of clinical characteristics: sex, age, tumor duration, tumor size, impacted teeth, root resorption, and metastasis to lymph nodes with BRAF^{V600E} IHC and PCR mutation examination.

		Ki 67 IHC					*BRAF IHC*			
Clinical characteristics	**n**	**positivef**	**negativef**	**OR**	***p* value**	**n**	**positivef**	**negativef**	**OR**	***p* value**
Sex	45									
Male		7 (15.6)	8 (17.8)	0.58	0.527	45	14 (31.1)	1 (2,2)	4.26	0.236
Female		18 (40)	12 (26.7)				23 (51.1)	7 (15.6)		
Age	45									
< 34 yrs old		15 (33.3)	8 (17.8)	0.44	0.236	45	20 (44.4)	3 (6.7)	0.51	0.459
≥ 34 years old		10 (22.2)	12 (26.7)				17 (37.8)	5 (11,1)		
Tumor duration										
< 12 years old	45					45				
≥ 12 years		7 (15.6)	4 (8,9)	0.643	0.729		8 (17.8)	3 (6.7)	2.17	0.382
		18 (40.0)	16 (35.6)				29 (64.4)	5 (11,1)		
Tumor size										
< 7cm	45					45				
≥ 7 cm		(35.6)	9(20,0)	0.460	0.239		20 (44.4)	5 (11,1)	1.42	0.716
		20)	11 (24.4)				17(37,8)	3 (6.7)		
Tooth impaction										
Yes										
No	45	11 (24.4)	7 (15.6)	1.46	0.760	45	15 (33.3)	3 (6.7)	1.14	1,000
		14 (31.1)	13 28.9)				22 (48.9)	5 (11,1)		
Root resorption tooth										
Yes	45					45				
No		13 (28.9)	16 (35.6)	0.271	0.066		25(55,6)	4 (8,9)	2.08	0.427
		12 (26.7)	4 (8,9)				12 (26.7)	4 (8,9)		
Invasion to KGB										
Yes	45	19 (42.2)	16 (35.6)	0.792	1,000	45	28 (62.2)	7(15,6)	0.44	0.661
No		6 (13.3)	4 (8,9)				9(20,0)	1 (2,2)		

Description: * Fisher's exact test, $p<0.05$ = significant

Table 3. Comparison of clinical characteristics with Ki-67 and BRAF^{V600E} IHC.

Table 3 showed no statistically significant ($p > 0.05$) relationships between clinical

characters; sex, age, tumor duration, tumor size, tooth impaction, root resorption, and metastasis to lymph nodes with Ki-67 and $BRAF^{V600E}$ IHC.

Relationship of tumor status (primary/recurrence) to ameloblastoma aggressivity

Table 4 shows that there is no statistically significant relationship (p>0.05) of tumor status (primary and recurrence) with ameloblastoma aggressivity: tumor location, tumor duration, invasion of surrounding tissues, lymph node invasion, root resorption, and results of aspiration, but there was a significant relationship (p<0.05 with the presence of impacted tooth. This table also shows impacted teeth were most found in primary ameloblastoma, 15 of 18 issues (33.3%), compared to 3 out of 18 samples (6.7%) in recurrence cases.

Variabel	Status Primary (%)	Tumor Rekurensi (%)	Nilai *p*
Tumor size			
< 7 cm	13 (28,9)	2 (26,7)	0,592
≥ 7 cm	12 (26,7)	8 (17,8)	
Location of tumor			
Maxilla	24 (53,3)	16 (35,6)	0,155
Mandibula	1 (2,2)	4 (8,9)	
Duration of tumor			
< 12 months	5 (11,1)	6 (13,3)	0,500
≥ 12 months	20 (44,4)	14 (31,1)	
Invasion to soft tissue			
No	12 (26,7)	8 (17,8)	
Yes	13 (28,9)	12 (26,7)	0,764
Invasion to lymph nodes			
No	4 (8,9)	6 (13,3)	0,301
Yes	21 (46,7)	14 (31,1)	
Impacted tooth			
Yes	15 (33,3)	3 (6,7)	0,003*
NO	10 (22,2)	17 (37,8)	
Tooth root resorption			
Yes	19 (42,2)	10 (22,2)	0,117
No	6 (13,3)	10 (22,2)	
Aspiration result			
Empty	4 (8,9)	2 (4,4)	
Red	16 (35,6)	16 (35,6)	0,615
Yellow	4 (8,9)	2 (4,4)	
Pus	1 (2,2)	0 (0,0)	

Description: Fisher's exact test, p<0.05 = significant

Table 4. Effect of tumor status on ameloblastoma aggressivity.

Relationship of the radiological features (unilocular/multilocular) with ameloblastoma aggressivity

Table 5 shows that there was no statistically significant relationship (p>0.05) of tumor status (primary and recurrent) with ameloblastoma aggressivity: tumor location, tumor duration, lymph node invasion, impacted tooth, root resorption, aspiration results, but there was a significant association (p<0.05) with the tumor size and tumor invasion into surrounding soft tissues.

Differences in radiological features to $BRAF^{V600E}$ expression, Ki-67 expression, and $BRAF^{V600E}$ mutation in ameloblastoma

Table 6 shows that there was no statistically significant relationship (p>0.05) of radiological features with $BRAF^{V600E}$ expression, Ki-67 expression, and $BRAF^{V600E}$ mutation in subjects with ameloblastoma.

Multivariate Analysis

$BRAF^{V600E}$ expression, Ki-67 expression, and $BRAF^{V600E}$ mutation, as well as oral hygiene status (OHIS) can be used as indicators of aggressive ameloblastoma (tumor size, tumor location, soft tissue invasion, lymph node invasion, tooth impaction, tooth root resorption, aspiration results) based on multivariate analysis.

Multivariate analysis was performed using logistic regression, which can simultaneously assess the association of an outcome with several risk factors. The multivariate analysis was performed by entering variables one by one, which in bivariate analysis has a p-value < 0.25. At the ratio of $BRAF^{V600E}$: Ki-67 expression 0.32, the obtained ROC was 0.51% (95% CI, 0.25%-0.75%) with 52.6% sensitivity, and 47.4% specificity.

Variables	Radiological Features		*p*
	Unilocular	Multilocular	
Tumor Size			
< 7 cm	8 (17.8)	17 (37.8)	0.005*
≥ 7 cm	0 (0,0)	20 (44.4)	
Location of the tumor			
Mandibula	7(15,6)	33 (73.3)	0.890
Maxilla	1 (2,2)	4 (8,9)	
Duration of the tumor			
< 12 months	2 (4,4)	9(20,0)	0.968
≥ 12 months	6 (13.3)	28 (62.2)	
Invasion to the soft tissue			
Not	7 (15.6)	13 (28.9)	0.015*
Yes	1 (2,2)	24 (53.3)	
Invasion to lymph nodes			
No	1 (2,2)	9(20,0)	0.661
Yes	7 (15.6)	28 (62.2)	
Impacted Teeth			
Yes	3 (6.7)	15 (33.3)	0.600
No	5 (11,1)	22 (48.9)	
Tooth root resorption			
Yes	6 (13.3)	23 (51.1)	0.400
No	2 (4,4)	14 (31.1)	
Aspiration Results			
Empty	1 (2,2)	5 (11,1)	
Red	6 (13.3)	26 (57.8)	0.970
Yellow	1 (2,2)	5(11,1)	
Pus	0 (0,0)	1(2,2)	

Description: * Fisher's exact test, p<0.05 = significant

Table 5. The effect of radiological features

(unilocular, multilocular) to the aggressivity of Ameloblastoma.

The multivariate analysis model of the risk factors for aggressive ameloblastoma is showed in Table 20. In that model, the impacted tooth variable is included in the multivariate model. Based on the Odd's ratio, the risk of a person with a $BRAF^{V600E}$: Ki-67 expression ratio of 0.32 to get aggressive ameloblastoma is 5.62 times greater than someone with a $BRAF^{V600E}$:Ki-67 expression < 0.32. Whilst the risk of someone with an impacted tooth is 0.71 times lower, but the results are insignificant.

Variables	Radiological Features		p
	Unilocular	Multiloculer	
$BRAF^{V600E}$ expression			
Low (2-3)	1 (2,2)	1 (2,2)	0,475
Intermediate (4-5)	1(2,2)	5 (11,1)	
High (6-7)	6 (13,3)	31 (68,9)	
Ki-67 expression			
Weak < 5	3 (6,7)	12 (26,7)	
Moderate 6-10	0 (0,0)	5 (11,1)	
High 11-20	1 (2,2)	2 (4,4)	0,652
Very high > 21	4 (8,9)	18 (40,0)	
$BRAF^{V600E}$ mutation			
Yes	3 (12,5)	16 (66,7)	0,270
No	2 (8,3)	3 (12,5)	

Description: * Fisher's exact test, p<0.05 = significant

Table 6. Differences in radiological features of $BRAF^{V600E}$ expression, Ki-67 expression, and $BRAF^{V600E}$ mutation in Ameloblastoma.

Clinical examination of aggressive ameloblastoma determined the predictive index of the $BRAF^{V600E}$: Ki-67 expression ratio, intending to facilitate the prediction of aggressive ameloblastoma through the model in Table 1. From Table 1 a linear regression equation is made as follows:

$P(y) = ß_0 + ß_1 X_1$

P (clinical prediction of *BRAF V600E expression ratio* : *Ki-67*) = 1.180 + (– 0.076) (OHIS)

The clinical model for the prediction of aggressive ameloblastoma is showed in Table 2. In that model, the combined variables of tumor duration and tooth impaction are still included in the final model because substantially these variables are important in the multivariate model.

The Odd's ratio value showed that the risk of someone with an OHIS of ≥ 3 to suffer from aggressive ameloblastoma was 3,364 times greater than someone with an OHIS < 3 index. The risk of someone with a tumor duration of ≥12 months to develop aggressive ameloblastoma was 2.72 times greater than that of < 12 months. The risk of a person who had an impacted tooth to develop an aggressive ameloblastoma was 0.857 times lower than that of a person who did not have an impacted tooth.

By using the beta coefficients of above model, the final model of the mathematical logistic regression equation is obtained as follows (from Table 2)

$$\text{Logit } P(y) = ß_0 + ß_1X_1 + ß_2X_2 + ß_3X_3$$

Logit P (aggressive ameloblastoma occurred) = 3,482 + 3,364 OHIS + 2,72 tumor duration + 0,857 impacted teeth.

The estimated probability for the occurrence of aggressive ameloblastoma is:

$$P = \frac{1}{1 + e^{-1}}$$

$$L = 3.482 + 3.36\ 4X_1 + 2.72\ X_2 + 0.857\ X_3$$

X1 = oral hygiene index
X2 = tumor duration
X3 = impacted tooth

The above equation states that aggressive ameloblastoma can be predicted clinically by means of OHIS, tooth impaction and tumor duration. The multivariate model above shows the risk score for aggressive ameloblastoma has a sensitivity of 47.1%, specificity of 50% and the area under the *ROC curve* is 0.475 (95% *CI* 0.282-0.667).

From the observation, it was found that the IHC $BRAF^{V600E}$ predicting mutations in the $BRAF^{V600E}$ PCR had a sensitivity of 73.7%, specificity of 60% and the area under the ROC curve was 0.658 (95% CI 0.30-0.935).

The next step is to create a clinical score against the risks of aggressive ameloblastoma defined in Table 3. Based on available data, the prediction of aggressive ameloblastoma is as follows:

Score < 8: low risk of aggressive ameloblastoma
Score 8-13: moderate risk of aggressive ameloblastoma.
Score > 13: high risk of aggressive ameloblastoma.

Discussion

Several variables that showed the aggressivity of ameloblastoma in this study were

tumor size, tumor duration, impacted tooth, tooth root resorption, lymph node metastasis, and soft tissue invasion. From the data obtained after analysis, the criteria for mild, moderate, and severe aggressive ameloblastoma were determined from clinical scoring, which had not been previously defined. These criteria can be used to establish a clinical diagnosis, determine a treatment plan, and establish a more accurate prognosis.

Compared to positive Ki-67 IHC expression which was only 57.8%, positive $BRAF^{V600E}$ expression in ameloblastoma samples was much higher at 82.2%. Similar high positive $BRAF^{V600E}$ expression in ameloblastoma samples had also been reported by several studies such as by Brown et al 2%, Diniz et al 82%, do Canto et al 78.57%, Kurppa et al 63%, Sweeney et al 46%, and Kelppe et al 72.2%.

The risk of someone with impacted tooth to have aggressive ameloblastoma is 0.857 times lower than those without impacted tooth.

By using the model beta coefficient, the final model mathematical logistic regression equation and the formula to calculate the probability of aggressive ameloblastoma are obtained. The equation shows that aggressive ameloblastoma can be clinically predicted through oral hygiene index, tooth impaction and tumor duration.

The application of these equations is still difficult, because the calculations are complicated so that only trained personnel can use them. Therefore, a scoring model that is more practical and easy to fill in and calculate is formulated.

A model that can be used by researchers and clinicians is shown in Table 4. Based on the available data, the prediction of aggressive ameloblastoma is as follows:
Score 0: mild risk of aggressive ameloblastoma
Score 10-20: moderate risk of aggressive ameloblastoma
Score > 20: high risk of aggressive ameloblastoma

Conclusions

The examination results in this study showed consistency between clinical examination and laboratory examination. It can be concluded that there is a relationship between $BRAF^{V600E}$ expression and ameloblastoma aggressivity. This study also produced a model to predict the aggressivity of ameloblastoma (mild, moderate, severe) based on oral hygiene status (score 1, score 2, and score >3, tumor duration, and the presence of impacted tooth). Examination of $BRAF^{V600E}$ immunoexpression, Ki-67 expression, and $BRAF^{V600E}$ gene mutation in this study can predict ameloblastoma aggressivity which is confirmed by clinical examinations of tumor duration, and tooth impaction.

The results of this study obtain a predictive model that can calculate individual's probability of getting aggressive ameloblastoma that can be used in daily practice. For this reason, $BRAF^{V600E}$ and Ki-67 IHC examinations can be considered to be routinely performed on ameloblastoma, in addition to assessing the clinicopathological aggressivity factors. Examinations of $BRAF^{V600E}$ and Ki-67 IHC are expected to be the markers of poor prognosis in in predicting the risk of ameloblastoma recurrences. Due to the associations of the clinical examination of aggressive ameloblastoma with $BRAF^{V600E}$ expression, Ki-67 expression, and $BRAF^{V600E}$ mutation, they can be used to strengthen the diagnosis by oral and maxillofacial surgeons and to assist the management of aggressive ameloblastoma in preventing recurrences. For the community, the results of this study can increase the knowledge about the effects of oral hygiene on aggressive ameloblastoma status, thus preventing ameloblastoma recurrences.

Declaration of Interest

The authors report no conflict of interest.

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